PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *THUNBERGIA FRAGRANS* Roxb. (ACANTHACEAE)

A dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University
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In partial fulfillment of the requirements
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26092034

DEPARTMENT OF PHARMACOGNOSY
COLLEGE OF PHARMACY
MADURAI MEDICAL COLLEGE
MADURAI - 625 020

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Prof. Mrs. R. THARABAI, M.Pharm.,
PRINCIPAL i/c,
College of Pharmacy,
Madurai Medical College
Madurai – 625 020.

CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *THUNBERGIA FRAGRANS* Roxb. (ACANTHACEAE)"** submitted by **Ms. S.LAKSHMI** in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University is a bonafide work done by her during the academic year 2010-2011 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai 625 020.

(R. THARABAI)

Dr. Mrs. AJITHADAS ARUNA, M. Pharm., Ph. D.,
Professor & Head,
Department of Pharmacognosy
College of Pharmacy,
Madurai Medical College
Madurai – 625 020.

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(AJITHADAS ARUNA)
Project Guide/Supervisor

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Dedicated To
My Beloved Family
Members, Teachers
And
Almighty

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INTRODUCTION

'Natural life' is the search of mankind in this 21th century. Ancient people were with nature and had strong health backbone (immune power). We people in the 21th century are in fully automated and mechanical world and thus the intimacy with nature has decreased and questionable too, this had presented a diseased life to mankind. Pharmacognosy is the branch of bioscience of treating diseases with the aid of nature's gift i.e. plants.

A single plant is a big industry manufacturing enormous active constituents. Research in plant is nothing but the act of deducing the active constituents in a scientific way. Man has yet to perceive those active principles that are manufactured in plants. Since they are the primary producers of food and medicines **each and every plant in the earth must be respected and protected** properly from natural disasters and urbanization.

Pharmacological actions of many herbs have not been scientifically validated; research is continuously being done to learn more about mechanism of action of plants on biological system. Strong standardization is needed for scientific acceptance of herbal medicines. We Indians still lack in those standardization parameters.

Significances of Medicinal Plants to Human Being [1]

Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin etc. Plants are directly used as medicines by a majority of cultures around the world, for example Indian Systems of Medicine and Chinese medicine. Many food crops have medicinal effects, for example garlic. Many of the diseases were treated effectively with herbal medicines and its leading molecules for example cancer.

Prevalent diseases in India [1]

Life style diseases are now killing more Indians than the infectious ones. WHO says India's disease pattern has undergone a major shift over the past decade. The latest WHO data paints a worrying picture that out of every 10 deaths in India, eight are caused by Non communicable diseases (NCDs) such as cancers, diabetes, cardiovascular diseases and chronic respiratory diseases in urban India. In rural India, six out of every 10 deaths is caused by NCDs.

A higher proportion (48%) of all NCD deaths occur in people under the age of 70 in developing nations like India compared with high-income countries (26%). Cancers were responsible for 27% of deaths, while diabetes was responsible for 4% of deaths.

Globally, 60% of the deaths are now caused by NCDs. Similar are the numbers in India. NCDs are affecting the entire globe. If not controlled, they will become a tsunami that will not only kill people but impair development and crash economies [1].

Common infectious diseases [2]

Among the common infectious mosquito born diseases are very common which include **Dengue, Malaria, Japanese encephalitis, influenza and yellow fever**. 50 million cases of dengue fever occur every year, it spread out by ***Aedes aegypti*** mosquito vector. In recent years Dengue has spread out all over Asia and Africa. ***Japanese encephalitis*** is a mosquito born disease caused by ***Culex tritaeniorhynchus,*** which is endemic in Asia. Around 50,000 cases occur each year, in that 25-30% of all cases are fatal. In case of malaria about 500 million people are affected annually. ***Anopheles stephensi*** is the vector

for malaria and it necessary to prevent these diseases in developing countries that face lots of economic problem.

Diabetes in India [3]

Diabetes is **not a disease**, **just a symptom** which leads to acute and long term complications, and the myriad of disorders associated with it, is a major health hazard in India. **India is becoming a diabetic nation** and it must be controlled with dietary management (Prevention before treatment). In keeping with the scenario of most developing countries, India has long passed the stage of a diabetes epidemic. The problem has now reached, in scientific language, "pandemic" proportions, it has crossed the dividing line and it is difficult to take lifelong allopathic treatment in developing nations due to economical problem. Since herbal medicines are economical than allopathic drugs, there is urge to control diabetes with natural medicines.

Current Status of Cancer in the world [3]

Cancer is the uncontrolled growth of abnormal cells in the body. Normal cells multiply when the body needs them, and die when the body doesn't need them. Cancer grows out of normal cells in the body and are out of control and cells divide too quickly. It can also occur when cells forget how to die. Many of the cancers are treated with herbal drugs. In fact many phytoconstituents acts as lead compounds for allopathic drug design.

Diabetes and Cancer [4]

Epidemiologic evidence suggests that people with diabetes are at a significantly higher risk for many forms of cancer. Type II diabetes and cancer share many risk factors than type I diabetes. Both cancer and diabetes are prevalent diseases whose incidence is increasing globally. Worldwide cancer is the 2^{nd} and diabetes is the 12^{th} leading factor for

death [1]. Cancer and diabetes are diagnosed in the same individuals more frequently; it indicates that there is some biological link between these diseases.

Biological Link between Diabetes and Cancer [5, 6, 7]

Carcinogenesis involves many steps which include initiation **(**irreversible first step toward cancer), promotion (stimulation of the growth of initiated cells), and progression (development of a more aggressive phenotype of promoted cells). Diabetes may influence the neoplastic process by several mechanisms, including **hyperinsulinemia** (either endogenous due to insulin resistance or exogenous due to administered insulin or insulin secretogogues), **hyperglycemia**, or **chronic inflammation**.

Insulin and insulin like growth factors (IGF) is a complex network of cell surface receptors. Most cancer cells express insulin and IGF-I; the A isoform of the insulin receptor is commonly expressed. This A isoform receptor plays a stimulative in insulin-mediated mutagenesis **[5].** The insulin receptor is also capable of stimulating cancer cell proliferation and metastasis. Since cancer cells uptake more of glucose continuously, the insulin receptor activation on neoplastic cells may relate more to cell survival and mitogenesis [6]. Many data shows when insulin level increase in blood due to high glucose, androgen synthesis in ovaries increases which leads to increased endogenous sex steroid levels and associated with high risk of endometrial cancer and breast cancer. Many cancer cells need high glucose (even glucose addiction), untreated hyperglycemia facilitates neoplastic proliferation.

The present work is aimed to studying the pharmacognostical phytochemical and pharmacological effects of *T. fragrans*. A special attempt has been made to study whether

the methanolic extract of plant possess any significant antidiabetic and anticancer properties as the incidence of diabetes and cancer occurring together has increased manifold.

AIM AND SCOPE

There is a need for scientific validation of plants that has been used in folklore medicine because of advancement of therapy and rapid deforestation that is taking place. The present work aims to scientifically validate *Thunbergia fragrans*, a climber which is fast becoming an endangered species.

PHARMACOGNOSTICAL EVALUATION

1. Collection and authentication of plant *Thunbergia fragrans* Roxb.
2. Description of plant based upon its macromorphology.
3. Microscopical evaluation of aerial parts of *T. fragrans* as the identification of plant.
4. Flouresence analysis of *T. fragrans* for both extract and crude powder form.
5. Development of standardization parameters as per the WHO 1996 guidelines as a preliminary step for the inclusion of *T. fragrans* to Ayurveda pharmacopeia.

PHYTOCHEMICAL EVALUATION

1. Extraction of *T. fragrans* aerial parts with various solvents.
2. Preliminary phytochemical screening of powder and various extracts of *T. fragrans.*
3. Quantization of phenol and flavanoid content in the methanolic extract of serial parts of the plant.
4. TLC and HPTLC finger profile for its methanolic extract as a preliminary step for authendification of *T. fragrans*.

PHARMACOLOGICAL EVALUATION

In vitro antidiabetic activity

To study the anti diabetic activity of *T. fragrans* by *in vitro* glucose diffusion model using dialysis membrane.

Antimicrobial activity

To study the antibacterial activity of the methanolic extract of *T. fragrans* on six commonly occurring bacteria and fungus.

In vitro anti inflammatory activity

To verify the folklore claim of *T. fragrans*, by *in vitro* anti inflammatory method (cell membrane stabilization).

Evaluation of antioxidant activity

To evaluate the antioxidant potential of *T. fragrans* by *in vitro* antioxidant methods

DNA damage protection study on plasmid DNA

1. **Culture of pBR322** *E.coli* strain on LB medium.
2. Preparation of various reagents for plasmid isolation alkaline lysis method
3. **Isolation of plasmid** by alkaline lysis method.
4. Verification of isolated plasmid DNA by running on Gel electrophoresis.

5. Evaluation of DNA damage protection efficiency of various concentrations *of T. fragrans* on isolated plasmid DNA by **Gel Electrophoresis**.

Anticancer activity of *T. fragrans* on uterine cancer cells

1. Culture of human uterine cancer cells (SiHa) in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin 20 IU ml^{-1}, streptomycin 15µg ml^{-1} and amphotericin 20 µg ml^{-1}).
2. Evaluating the antiproliferative effect of *T. fragrans* methanolic extract by **MTT assay**.
3. **Microscopic analysis of treated cell lines** for anticancer activity.

The present investigation on *Thunbergis fragrans* will fill up the lacuna in our knowledge of the plant. It is also aims in providing botanical standardization, screening for potential antidiabetic and anticancer activities of the extract and correlation of the activity with phytoconstituents.

REVIEW OF LITERATURE

A detailed review of literature from primary and secondary sources on the genus *Thunbergia* is described in this chapter. Paucity of information available on the pharmacology, and phytochemistry on *T. fragrans* formed the basis of the study.

Pharmacology:

Manoj GG *et al.*, [1993] have done clinical study on T. *fragrans* in hepatic diseases [8].

Aritajats *et al.*,[2004] have studied the anti diabetic activity of *T. laurifolia* Linn. in rats. The effects on the reproductive system and the histology of the pancreas were also investigated. 60mg/ml/day extract was given to animal, 15-day treatment *T. laurifolia* extract decreased levels of blood glucose in alloxan induced diabetic rats. They proposed that the leaf contains insulin like substances which directly acts as hypoglycemic agents [9].

Pramyothin *et al.*, [2005] have studied the hepatoprotective activity of *Thunbergia laurifolia* Linn. Extract [TLE] both in vitro & in vivo models. The primary cultures of rat hepatocyte were used for in vitro study. In the in vitro study, MTT reduction assay & release of transaminases [ALT & AST] were observed for cell viability. Silymarin (SL) was the reference hepatoprotective agent used. Both TLE, SL increased MTT. Ethanol (4g/kg day for 14 days) caused increased level of AST, ALT, HTg & Centrilobular hydropic degradation of hepatocytes.TLE (25 mg/kg day) & SL (5mg) for 7 days after ethanol enhanced liver cell recovery [10].

Shekhar SD *et al.*, [2007] have studied the antidiarrhoeal activity of acetone & ethonolic extracts of *Thunbergia fragrans* Roxb. leaves on GI Motility by using Swiss albino mice. The antidiarrhoeal activity was given significant at 500mg/kg body weight in charcoal meal model [11].

Oonsivilai R *et al.*, [2007], have done phytochemical profiling and phase II inducing properties in *T. laurifolia* Linn (Rang cheut). *T. laurifolia* extract in water, ethanol, acetone were prepared and phytochemical profile was done. The total phenolic content was found to be 24.33, 5.65, 1.42 mcg GAE. *T. laurifolia* exhibited strong dose dependent antimutagenic activity in 2-amino anthracene induced mutagenics upto 87% in *Salmonella typhimurium* [12].

Oonsivilai R *et al.*, [2008] have reported the antioxidant activity and cytooxicity of *T. laurifolia* water, ethanolic and acetone extracts. Ferric reducing antioxidant power assay (FRAP) and Folin ciocalteu method were carried out for free radical scavenging activity. Water extracts showed the highest total anti oxidant activity using FRAP assay at 0.93mg/g. cytooxicity of *T. laurifolia* crude extracts were investigated in L929, BHK (21)C13, Hep, H2 & Ca CO-2 cell lines. The toxicity was indicated at high concentration over 100mg/mL for all extract [13].

Palipoch S *et al.*, [2011] have studied the protective efficiency of *T. laurifolia* leaf extract against lead (II) nitrate induced toxicity in *Oreochromis niloticus*. 6 groups of fish were treated with 40ppm of waterborne lead (II) nitrate and 50% ethanol extract for 28 days. Peripheral blood and organs were collected, blood chemistry and hematology and

histology were investigated. *T. laurifolia* leaf clearly reduced the toxicity and is able to promote growth performance in *O. niloticus* after $PbNO_3$ exposure [14].

Phytochemistry

Jensen SR *et al.*, [1989] isolated iridods from *Thunbergia* species. Among the species of Thunbergia, 4 contain stilbericoside a iridoid glycoside, in addition 6- epi-stilbericoside. Another one had thunbergioside [15].

Ismail LD, *et al.*, [1996] have isolated novel iridoid glycosides, isounedoside and grandifloric acid from *T. grandifolia.* Grandifloric acid contains C-10 as a carboxylic acid group, which was predicted by recent iridoid biosynthesis studies carried out within *T.alata.* NMR spectral assignments for the known iridoid glycoside alatoside were also made [16].

Frederiksen LB *et al.*, [1999] explained the biosynthesis of iridoids lacking C10 in *T. alata*. Deuteriam labeled 6-deoxyretzioside was incorporated into stilberoside in *T. alata*. Chloroplast DNA Sequencing showed a special kind of iridoid & it explained the close relationship between Nuxia, Retzia & stilberaceae. A similar relationship was found berween Hyderangeacea & Loasaceae[17].

Phore, MM *et al.*, [2000] isolated amino acids & sugars from floral nectors of *T. erecta* & some other plants. 7 aminoacids were insolated from the nectar of *T. erecta*. Fructose was the most common sugar and glutamic acid was found to be most common amino acids [18].

Kanchanapoom T *et al.,* [2002] isolated two iridoid glycosides from extract of aerial part of *T. Laurifolia* along with 7 unknown compounds. The structural elucidation was based on the analysis of spectroscopic data [19].

Dash SS *et al.,* [2009] have done a survey of useful plants of kabi sacred grove, Sikkim. Traditional plants used for treatment were listed out including *Thunbergia lutia* [20].

Miscellaneous

Damtoft S *et al.,* [1994] in their review article that contains 90 hemi – and 188 monoterpenoid glycosides have suggested that some of these compounds possess antioxidant & anti cancer antimicrobial & antibacterial properties. Among those glycosides alatoside & thunaloside (iridoid glycosides) were found from *Thunbergia alata* [21].

Rajah G *et al.,* [2002] made a new record of somatic chromosome numbers in two species of Acanthaceae. In *T. tomentosa* & *Gold fussa isophylla* chromosome number was found out, Karyophyte analysis was done and histogram drawn to show comparative amount of DNA [22].

PLANT PROFILE

THUNBERGIA FRAGRANS

Family: ***ACANTHACEAE*** **(**[23].

Latin name: *Thunbergia fragrans* Roxb. **(**[23].

Synonym: *Thunbergia volubilis* Pers, *Flemingia grandiflora* Roxb. ex Rottler [24]

Common name: White lady, white thunbergia, sweet clock-vine , white clock-vine.[23, 25]

Systematic position

Kingdom	– *Plantae*
Sub kingdom	–*Tracheobionta*
Super division	– *spermatophyta*
Division	– *Magnoliophyta*
Class	– *Magnoliosida*
Sub class	– *Asteridae*
Order	– *Scrophulariales*
Family	– *Acanthaceae*
Genus	– *Thunbergia*
Species	– *fragrans*

Common vernacular name

Hindi	: Chimine
Malayalam	**:** Noorvanvilli
Marathi	: Chimine
Tamil	: Indrapushpam

Geographical source

T. fragrans is native to India and Ceylon [23]. *T. fragrans* is naturalized in tropical and subtropicals regions of the world. According to PIER (2003), *T. fragrans* is present in the Cook Islands (Rarotonga, Mangaia), Federated States of Micornesia (Pohnpei), Fiji, Guam, Hawai'i, New Caledonia, Niue, Samoa (Upolu, Savai'i), Tonga (Tongatapua, 'Eua, Vava'u, Lifuka/Foa, Ha'ano), Vanuatu, China (native), and Indochina (native). In the United State, *T.fragrans* is known from Florida, Hawai'i, Puerto Rico and the Virgin Islands [26].

Taxonomic notes:

The genus *Thunbergia* is made up of about 200 species from warm areas of central and southern Africa, Madagascar, and Asia [23].

Nomenclature:

The genus is named after the Swedish botanist and explorer, Carl Peter Thunberg (1743-1822) [27].

Other species:

Other naturalized species include *T. alata* (black-eye Susan vine), *T. grandiflora* (blue trumpet vine), and *T. laurifolia* (purple allamanda). A few *Thunbergia* species are cultivated but are not yet naturalized, including *T. erecta* (king's mantle) and *T. mysorensis* (mysore clock vine).

Propagation:

T. fragrans is propagated from seeds, cuttings, and fragments of roots [28].

Ethnomedical information for whole plant [29]

Whole plant extract was used in inflammatory conditions.

PHARMACOGNOSTICAL EVALUATION [30, 31]

Crude drugs must be evaluated for its identity, quality, purity and detection of adulterants. Many evaluating procedures include macromorphalogical, microscopical, physical, chemical and biological evaluations. With advent of separation techniques and instrumentation analysis, it is possible to perform physical evaluation of a crude drug which is for both qualitative and quantitative nature. The process of evaluation is termed as standardization.

Organoleptic evaluation [30]

It refers to the evaluation by means of using our sense of organs for detecting color, odour, size, shape and special features including touch, texture etc., It gives the majority of information on identity purity, quality of crude drugs.

Macromorphological Evaluation

Morphology is the study of the form of object. Macro morphology (or) grass morphology is study and description of the whole drug.

Microscopical Evaluation [30]

This is the identification of organized drugs by their known histological characters. It is mostly used for the qualitative examination of organized drugs both in entire and powder forms. By using magnifying property of microscope, minute structures are enlarged and structural details are confirmed. Various reagents and stains can be used to distinguish cellular structures.

MATERIALS AND METHODS

MACROSCOPICAL AND MICROSCOPICAL EVALUATION

Collection of Specimens

The plant specimens were collected from in and around Madurai Medical College, Madurai-20. The specimen was authenticated by Prof. P. Jayaraman, Director, Plant Anatomy Research Centre, West Tambaram, Chennai. A plant specimen was kept in the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai. Precaution was taken to select healthy plants and normal organs to avoid diseased plants. Different organs from the plant were cut and fixed in FAA (formalin, 5mL; acetic acid, 5mL; ethyl alcohol, 90mL). The specimens were dehydrated After 24 hrs of fixing with graded series of t-butyl alcohol [32]. Infiltration of the specimens was carried out by addition of paraffin wax gradually (M.P-58-60^{o}C) until TBA solution attained super saturation. The specimens were cast in to paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with help of rotary microtome. The thickness of the sections was 10-12 µm. De-waxing of the sections was carried out by customary procedure [33]. The sections were stained with toluidine blue [34] since it is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. Cellulose walls were pink, lignified cells and protein were blue and mucilage taken violet color.

Leaf clearing

Two methods were used for studying the stomatal morphology, venation pattern and trichome distribution. Paraffin embedded leaf was prepared and sectioned and the epidermal layers as well as vein islets were studied. Another method employed was immersing the material in alcohol (to remove chlorophyll) the leaf for clearing fragments by followed by treating with 5% sodium hydroxide. The material was transparent due to loss of cell contents. Epidermal peeling by partial maceration employing Jeffrey's maceration was also done. Glycerin mounted temporary preparations were made for cleared materials.

For study of elements of xylem, small fragments of stem and root were macerated with Jeffery's maceration fluid.

Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component were studied and measured.

Photomicrographs

Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observations bright field was used and for the study of lignified cells and starch grains, polarized light was employed. Structures appear bright against dark background due to bi-refringent property.

The photographs of macroscopical characters and the photomicrographs of microscopical analysis are presented in **Figs. 1 to 10.**

QUANTITATIVE ANALYTICAL MICROSCOPY [35, 36]

This is technique of measurement of cell contents of the crude drugs and thus help in their identification, characterization and standardization. A clear idea about the identity and characteristic features of the drug can be obtained after several numbers of determinations.

Determination of Leaf Constants

The vein islet number, vein termination number, stomatal number, stomatal index and palisade ratio were determined on fresh leaves using standard procedure.

Stomatal Number

Stomatal number is the average number of stomata per square mm area of epidermis of the leaf.

Determination of Stomatal Number

A piece of upper and lower epidermal peelings was mounted on a slide with the help of camera lucida and stage micrometer 1 mm square was drawn on black chart. The stage micrometer was replaced with the preparation. Then the preparation was observed and the stomata marked in that unit area. Ten such readings were taken and the average of stomatal number was calculated and reported in the **table 1.**

Stomatal Index

Stomatal index is the percentage which the numbers of stomata to the total number of epidermal cells each stomata being counted as one cell.

Stomatal number= S/S+E.; S- Number of stomata per unit area and E- Number of epidermal cells in same unit area.

Determination of Stomatal Index

The procedure adopted in the stomatal number was followed and then the preparation was observed under high power. The epidermal cells and the stomata were counted. From these values stomatal index was calculated from the formula and tabulated in **table 1**.

Vein Islet Number and Vein Termination Number

The term vein islet is used to denote the minute area of photosynthetic tissue encircled by the ultimate division of the conducting strands. The number of vein islets per square mm area is called vein-islet number.

Vein termination number is defined as the number of vein termination present in one square mm area of the photosynthetic tissue.

Determination of Vein Islet and Vein Termination Number

The fresh leaves were cut into pieces from various regions between midrib and margin, cleared in chloral hydrate and mounted on a slide. With the help of stage micrometer, camera lucida and microscope 1mm square was drawn on the black chart. The stage micrometer was replaced by preparation and veins were traced in that square. Vein islet and vein termination was counted from the square drawn. Ten such readings were done and average was calculated. The results obtained in the number of vein islets and terminals in1 sq mm were tabulated in **table 1**.

Determination of Palisade ratio

Palisade ratio is the average number of palisade cells beneath each epidermal cell. It is one of the important criterion for identifications and evaluations for crude drugs since it is constant for a plant species which is useful to differentiate the species and does not altered based on geographical variation [35, 36].

The epidermis was peeled and partial maceration done by Jeffery's maceration fluid [32]. A fragment was transferred into a microscopical slide and the mount of upper epidermis was prepared on one side of the cover slip to prevent the preparation from drying. The same was examined under 45X objective and 10X eye piece. Four adjacent epidermal cells were traced and the palisade cells under the epidermal cells were counted and the palisade ratio was calculated by using the following formula and the results were tabulated in **table 1.**

Palisade ratio = Avg. number of palisade cells beneath the 4 epidermal cells/4.

POWDER ANALYSIS

The behavior of the powder with different chemical reagents was carried out as mentioned by Kay (1938) and Johansen (1940) [37, 33]. The observations are presented in **table 2.**

Fluorescence analysis

The fluorescent analysis of the drug powder as well as the plant extracts of *T. fragrans* were carried out by using the method of Chase and Pratt (1949) [38]. The observations are tabulated in **tables 3 and 4.**

STANDARDIZATION PARAMETERS

Ash values, loss on drying (LOD), foreign organic matter and extractive values etc., gives a clear idea about the specific characteristics of crude drug under examination, besides its macro-morphological or cyto-morphological, microscopical nature in both its entire and its powder form. The procedures recommended in Indian Pharmacopoeia, 1996 and WHO guidelines, 1998 [39, 40] were followed to calculate parameters.

Determination of Volatile oil [35, 40]

Volatile oils are low molecular weight compounds which easily volatile in room temperature. They have many biological actions. They chemically consist of monoterpenes, sesquiterpenes and their oxygenated derivatives.

100g of fresh plant material was crushed and introduced into the flask which contains distilled water until the one third of plant material was immersed in water. Few porcelain bits were added and heated for 3h. Volatile oil content was noted in the graduated receiver. Oil content of the plant material was calculated in mL/100g of plant material and the result was reported in the **table 5.**

Determination of foreign Organic matter [39, 40, 41]

The part of organ or organs other than those specified in the definition or description of the crude drugs is defined as foreign organic matter. The maximum limit for the foreign organic matter is defined in the monograph of crude drugs.

100g of air dried powdered drug was spread out in a thin layer. The sample drug was inspected with the unaided eye or with the use of 6X lens and the foreign organic

matter was separated manually and weighed. The percentage of foreign organic matter was calculated with reference to the weight of the drug taken. The result was reported in **table 5.**

Determination of Moisture Content (Loss on Drying) [40]

Amount of water and volatile oil present in the specified quantity of plant material is known as loss on drying. Medicinal; plant materials should not contain excess of water because; it encourages microbial growth and detoriation followed by hydrolysis.

One gram powder was accurately weighted in a tarred petri dish, previously dried under the conditions specified in IP'96. Powder was distributed evenly by gentle sidewise shaking. The dish was dried in oven at 100-105^{o}C for one hour. It was cooled in a dessicator and again weighed. The loss on drying was calculated with reference to the air dried powder.

Determination of Ash Values [39, 40]

The residue remaining after incineration of plant material is known as ash content of the crude drug, which simply represents the inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it, as a form of adulteration. The object of ashing vegetable drugs is to remove all traces of organic matter which may otherwise interfere in an analytical determination. Total ash contains both physiological and nonphysiological ash. Physiological ash consists of the plant tissues, while the nonphysiological ash consists of residue of extraneous matter (such as sand, soil etc.,) adhering to herb itself.

Ash value is useful for detecting low grade products, exhausted drugs and excess of sandy or earthy matter; it is more especially applicable to powdered drugs. Ash values were determined as per official method using air dried material.

Total Ash

Two grams of air dried powder was accurately weighed in a silica crucible, it was spread fine even layer on the bottom of crucible and incinerated gradually increasing the temperature not exceeding 450^{o}C until free from carbon, then cooled and weighed for constant weight. The percentage of ash with reference to the air dried powder was calculated.

Water Soluble Ash

The total ash obtained above was boiled with 25ml of water for 5 minutes and the insoluble matter was collected on an ashless filter paper. Then it was ignited for 15 minutes not exceeding 450^{o}C. Water insoluble ash was subtracted from total ash weight. It represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.

Acid insoluble ash

The total ash obtained was boiled with 25mL of dilute hydrochloric acid. The acid insoluble matter was collected in a silica crucible. The residue obtained was dried and weighed. The percentage of acid insoluble ash with reference to air dried material was calculated.

The values in respect of the total ash values, acid insoluble ash, water soluble ash and water insoluble ash are tabulated in **table 5.**

Determination of Extractive Values [39, 40, 41]

This is the method of determining the amount of active principles extracted with solvents from a given amount of plant material. Extractive value is indicative of active constituent that is soluble in particular solvent. The solvent used fro extraction should dissolve appreciable quantities of substances desired.

Alcohol (Ethanol) Soluble Extractive Value

Five grams of air dried drug coarsely powdered, was macerated in 100ml of ethanol in a closed flask for 24h, shaking frequently 6h and allowed to stand for 18h, filtered rapidly, taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish at 105^{o}C, to constant weight. The percentage of alcohol soluble extractive with reference to the air dried drug was calculated and represented.

Methanol Soluble Extractive Value

Five grams of air dried drug coarsely powdered, it was macerated with 100mL of methanol for 24h in a closed flask, frequently shaking 6h and allowed to stand for 18h. Filtered rapidly, taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred shallow dish at 105^{o}C, to constant weight. The percentage of methanol soluble extractive with reference to the air dried drug was calculated.

Water Soluble Extractive Value

5g of air dried coarsely powdered drug material was macerated with 100mL of water for 24h, frequently shaking for 6h and allowed to stand for 18h. Filtered rapidly, taking precautions against loss of solvent. 25mL of filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105^{o}C to constant weight. The percentage of water soluble extractive with reference to air dried drug was calculated.

Petroleum ether soluble extractive value

The procedure adopted under ethanol soluble extractive was followed using petroleum ether as a solvent.

Ether soluble extractive value

The procedure adopted under ethanol soluble extractive was followed using diethyl ether as a solvent.

Chloroform soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using chloroform as a solvent.

Ethyl acetate soluble extractive:

The procedure adopted under ethanol soluble extractive was followed using ethyl acetate as a solvent.

The extractive values obtained for different solvents are presented in **table 5.**

Determination of Foaming Index [40]

Plant materials which contains saponins will produce persistent foam when they shaken with water. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.

1g of powdered material was taken in 500mL of conical flask with 100mL boiling water. It was allowed to boil for 30mins then cooled and filtered. Sufficient volume of water added to produce 100mL filtrate. This filtrate was poured in 10 stoppered test tubes in successive portions of 1mL, 2mL, 3mL etc., upto 10mL, volume was adjusted to 10mL in each test tube, shaken lengthwise for 15 sec, 2 shakes per second. Allowed to stand for 15mins and the height of foam was measured. If the height of the foam in every tube was less than 1cm the foaming index was less than 100. If a height of foam of 1cm was measured in any test tube, the volume of the plant material decoction in this tube (a) was used to determine the index. If this tube was first or second tube in a series, an intermediate dilution was prepared in a similar manner to obtain a more precise result.

If the height of the foam was more than 1cm in every tube, the foaming index was over 1000. In this case, the determination was repeated using a new series dilution of the decoction in order to obtain a result. The foaming index was calculated using the following formula 1000/A where A was the volume in mL of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed. The result obtained is presented in **table 5**.

Determination of Swelling Index [40]

Swelling index is the volume in mL taken up by the swelling of 1g of plant material under specified conditions.

Medicinal plant materials which have gums, mucilage and pectin or hemicellulose will have pharmacological and pharmaceutical activity due to the swelling property.

1g of powder was taken in a 25mL glass stoppered measuring cylinder. Water added upto 25mL and shaken every 10mins for 1hr, followed by standing 3h at room temperature. Volume occupied by the plant material including sticky mucilage was noted. The results obtained are presented in **table 5.**

OBSERVATIONS

MACROMORPHOLOGICAL OBSERVATIONS

Thunbergia fragrans Roxb. is a vigorous, woody vine from India that has a variable height and width, depending on the size of the structure on which it is growing. This plant produces rounded seed capsules that end in a beak and has attractive lovely flowers.

Leaves (Fig.1. 6, 7)

The leaves hastate-ovate, 4-10 cm long, 3-5 cm wide, margins subentire. The leaves are small, triangular-ovate that has entire to finely toothed margins. The leaves has stalk, simple, opposite, sub sessile, hastate or ovate, 2-10cm long, 3-5 cm wide, pointed at the tip and heart shaped at the base. Young leaves are dull green color and mature leaves are dark green in color.

Flowers (Fig.1.3)

Thunbergia fragrans has attractive flowers, subtended by 2 lanceolate to ovate bracts 13-15 mm long; calyx lobes 15-16; corolla white, 1.5-3 cm long. Flowers are white in colour

Fruit (Fig. 1.4)

The fruit resembles a bird's head it has rounded seed capsules that end in a beak.

Root

The roots are cylindrical shape and it has brownish black color in external, white an internal also it has fibrous fracture.

MICROSCOPICAL OBSERVATIONS

Leaf (Fig. 2.1, 2)

The leaf consists of a thin midrib with small, short adaxial conical part and short semicircular abaxial part **(Fig 2.1)**. It is 60µm thick and the vascular bundle of the midrib is triangular with compact parallel rows of thick walled xylem elements and is of isolated small nests of phloem elements. A thin layer of highly thick walled fibers is seen beneath the phloem **(Fig. 2.2).**

Lamina (Fig. 3.1)

The lamina is 170 µm thick; the adaxial epidermis is thick with large rectangular cells measuring 20 µm thick. The cuticle is prominent; the abaxial epidermis is comparatively thin, comprising narrow cylindrical cells. The palisade zone includes the single row of thick cylindrical columnar palisade cells. The spongymesophyll consists of 4 or 5 layers of large, lobed parenchyma cells. The vascular strand of the lateral vein has a

vertical thin row of xylem elements and a small group of phloem. The vascular strand has a single ring of dilated parenchymatous bundle sheath.

Venation (Fig 3.2, 6.1)

The venation is less dense and the vein islets are not well demarcated by vein boundaries. However, the vein termination are well defined and mostly branched. The veins are always associated with bundle sheath parenchyma.

Crystals (Fig 4.1-3; 6.1-3)

The bundle sheath parenchyma cells have dense accumulation of short, spindle shaped needles of calcium oxalate. In T.S of the leaf, the crystal having sheath cells are seen in a circle around the vascular bundles **(Fig 4.1-3).** In paradermal sections the crystalliferous sheath cells are seen all along the path of branched veins **(Fig 6-1-3).**

Epidermal cells and stomata (Fig 5.1,2,3)

The adaxial epidermis is apostomatic, the **cells are highly lobed and amoeboid** in outline due to wavy anticlinal walls **(Fig. 5.1).** The abaxial epidermis is densely stomatiferous **(Fig 5.2,3).** The **stomata are diacytic type**, the guard cells are elliptic oblong and are 30x40 µm in size .The epidermis cells are similar to the adaxial cells is having wavy anticlinical walls and amoeboid outline.

Petiole (Fig. 7.1, 2)

The petiole is planoconvex in sectional view with flat adaxial side. It consists of a thin epidermal layer and parenchymatous ground tissue. The vascular strands comprise a discrete deep are of three larger collaterall bundles at the ends of the arc **(Fig. 7.1).** The

bundles have compact long rows of thick walled lignified xylem elements and a thin arc of phloem elements **(Fig 7.2).**

Stem (Fig. 8.1, 2, 3)

The stem is 4 angled and four winged in sectional view. A thin layer of epidermis and fairly wide parenchymatous cortical zone are seen. The pith is wide and parenchymatous. The vascular cylinder is a closed with thin thickened corners. In the four corners of the cylinder are seen a small group of wide circular vessels. In the remaining portions the cylinder is comprising thick walled fibers **(Fig 8.2,3)**.The vessels are 60-90 µm wide.

Powder microscopy

The following inclusions are seen in the powder: **Adaxial epidermal peelings** are seen which have no stomata. The anticlinal walls are highly wavy and the cells amoeboid in shape **(Fig 9.1)**.

Abaxial epidermal peelings (Fig 9.2,3) with dense stomata are frequently seen in the powder. As in the adaxial epidermis, the epidermal cells are lobed and amoeboid. The stomata are diacytic type with subsidiary cells at right angles to the guard cells **(Fig 9.3).**

Nonglandular epidermal trichomes are abundant in the powder **(Fig. 10.1, 2).** The trichomes are 2 or 3 celled, unbranched, thick at the base and pointed at the tip. The walls are thick .No cell inclusions are seen **(Fig 10.2)** .The trichomes are 250micrometer thick.

Crystals: Spindle shaped needle crystals of calcium oxalate are abundant. They are scattered and are seen isolated. Squarish crystals are also frequently seen in the powder **(Fig 10.3).**

QUANTITATIVE ANALYTICAL MICROSCOPY

The observations in respect of stomatal number and index, palisade ratio etc. are presented in the table 1.

Table 1: Quantitative analytical microscopical parameters of the leaf of *T.fragrans*

S. No.	Parameters*	Values obtained
1	Stomatal number in upper epidermis	55.1 ± 1.55
2	Stomatal index in upper epidermis	0.33 ±0.01
3	Vein islet number	2.4 ± 0.53
4	Vein termination number	7.3± 0.68
5	Palisade ratio in upper epidermis	4.0 ± 0.58

*** mean of 6 readings ± SEM**

POWDER ANALYSIS

The behavior of *T .fragrans* with various chemical reagents was tabulated **(table 2).** Fluorescence analysis results are given in **table 3 and 4.** The powder showed the presence of phyto sterols, proteins, starch, flavonoids and tannins.

Table 2: Behavior of the *T. fragrans* powder with various chemical reagents

Powder + Reagents	**Color / Precipitate**	**Presence of active principle**
Picric acid	Yellow precipitate	Protein present
Conc. sulfuric acid	Reddish brown color	Phyto sterols present
Lieberman Burchard reagent	Reddish brown color	Phyto sterols present
Aqueous ferric chloride	Greenish black color	Tannins present
Iodine solution	Blue color	Starch present
Mayer's reagent	No cream color	Absence of alkaloids
Spot test	No stain	Fixed oils absent
Sulfosalicylic acid	White precipitate	Protein present
Aq. Sodium hydroxide	Yellow color	Flavonoids present
Mg - HCl	Magenta color	Flavonoids present
Aq. Lead acetate	White precipitate	Tannins present

Note :- Color reactions are viewed under natural light by naked eye

Table 3: Fluorescence Analysis of powder of *T. fragrans*

Powder +reagent	**Day light**	**UV light (254 nm)**	**UV light (366 nm)**
Drug powder	Light green	Light green	Brownish green
Drug powder +aqueous 1M sodium hydroxide	Dark green	Dark green	Dark green
Drug powder + alcoholic 1M sodium hydroxide	Dark green	Dark green	Dark green
Drug powder + iodine	Bluish green	Bluish green	Brownish red
Drug powder + 10% potassium hydroxide	Green	Dark green	Brownish red
Drug powder + 1M hydrochloric acid	Yellowish green	Dark green	Dark brown
Drug powder + glacial acetic acid	Yellowish green	Dark green	Reddish orange
Drug powder + 50% sulphuric acid	Brownish green	Dark green	Dark gray
Drug powder + 50% nitric acid	Brown	Dark green	Dark gray
Drug powder + 50% hydrochloric acid	Grayish brown	Dark green	Grayish brown

Table 4: Fluorescence Analysis of extracts of *T. fragrans*

Extracts	**Day light**	**UV light (254 nm)**	**UV light (366 nm)**
Petroleum extract	Light green	Light green	Reddish orange
Ether extract	Light green	Light green	Reddish orange
Chloroform extract	Dark green	Dark green	Reddish orange
Ethyl acetate extract	Light green	Light green	Reddish orange
Ethanol extract	Dark green	Dark green	Reddish orange
Methanol extract	Fluorescence like	Fluorescence like	Reddish orange
Aqueous extract	Dark green	Dark green	Brownish orange

The **table 4** shows that the methanolic extract was reddish orange on UV light at 366 nm and flouresence like color in 254 nm.

STANDARDISATION PARAMETERS

The results obtained for various standardization parameters are presented in **table 5.**

Table 5: Standardization parameters of *T. fragrans*

S. No	**Parameters***	**Values* expressed as %**
1	**Volatile oil**	Nil
2	**Foreign organic matter**	0.01± 0.001
3	**Moisture content**	0.03 ± 0.014
4	**Ash values**	
	Total ash	20.52 ± 5.92
	Acid insoluble ash	0.264 ± 0.008
	Water soluble ash	3.76 ± 0.0432
	Water insoluble ash	0.337 ± 0.011
5	**Extractive Values**	
	Petroleum extract	0.030 ± 0.002

	Ether extract	0.027 ± 0.003
	Chloroform extract	0.066 ± 0.006
	Ethyl acetate extract	0.038 ± 0.003
	Ethanol extract	0.088 ± 0.016
	Methanol extract	0.205 ± 0.011
	Aqueous extract	0.418 ± 0.019
6	**Foaming index**	Nil
7	**Swelling index**	**Expressed as mL**
	Initial volume	7.5 ± 0.76
	Final volume	9.16 ± 0.927

*** mean of three readings**

DISCUSSION

MICROSCOPICAL EVALUVATION

Microscopical evaluation showed the cell types and cell inclusion details. The leaf was apostomatic with diacytic stomata, non glandular epidermal trichomes and midrib was small with short adaxial side and a short semicircular abaxial part. It had a single row of thick cylindrical columnar palisade cells. The venation was less dense and not well demarcated by vein boundaries. The vein terminations were well defined and the leaf had a planoconvex petiole.

The microscopic studies on the stem showed four angled and four winged in sectional view. It also showed the vascular cylinder which had thickened corners. The cylinder is four comprising thick walled fibers vessels are 40-90 µm wide.

Evaluation of powder microscopy showed abundant non glandular epidermal trichomes 250µm long and 20µm thickness and the abaxial epidermal fragment has diacytic stomata 30x40 µM in size with subsidiary cells at right angles with graved cells; spindle shaped needle crystals of calcium oxalate were observed. Squanish crystals are also frequently seen in powder.

The observed macroscopical, microscopical features will play in the authenticity of the plant *T .fragrans*.

POWDER ANALYSIS

Evaluation of the powder with various reagents provides information about the presence or absence of phytoconstituents which is useful to identify certain characteristics of the plant *T. fragrans*.

QUANTITATIVE ANALYTICAL MICROSCOPY

Quantitative analytical microscopy gives constant ranges of stomatal number, index and vein termination, vein islets number and palisade ratio which can be utilized for the authentication of crude drug in future. These quantitative analytical microscopy values remains constant for a given plant species and it will not get altered by geographical variation and also differ from other plant species.

STANDARDISATION PARAMETERS

The standardization parameters like ash values, extractive values, moisture content etc., were can be used to ensure the purity, quality of the herbal drugs. The ash value represents both organic, inorganic matter present in the crude plant material.

The powdered form of herbal drugs is more convenient form for easy handling and storage. In powder form herbal drugs are more susceptible for adulteration, spoilage and insect infestation. The standardization parameter and powder analysis give a clear view about its purity, quality and other distinguishable characters of *T. fragrans* from other species.

PHYTOCHEMICAL EVALUATION

Phytochemistry deals with natural product organic chemistry and plant biochemistry. It also deals with a variety of secondary metabolites that are produced by plants, their chemical structures, biosynthesis, metabolism, natural distribution and biological functions**.** For these operations, methods are needed for separation, purification and identification of the many different constituents present in plants [41].

MATERIALS AND METHODS

QUALITATIVE CHEMICAL TESTS FOR CRUDE EXTRACTS [35, 36, 41]

The chemical nature, specific identity, polarity, etc of the substances in the crude extract can be determined by a number of ways including wet chemical tests. In that a color reaction or precipitate is response to specific compound usually a class of compound. Such test can be useful for the investigation of the chemical compounds and to monitor the effectiveness of an extraction process. The petroleum ether, ether, ethyl acetate, methanol, ethanol and aqueous extracts were subjected to qualitative chemical analysis. The various tests performed on the extracts were for steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, quinones, phenols, tannins and saponins and the results were tabulated.

1. Test for steroids

Liebermann Burchard test: 1mg of the test substance was dissolved in a few drops of chloroform, 3ml of acetic anhydride and 3ml of glacial acetic acid were added, warmed and cooled under the tap and drops of concentrated sulphuric acid was added along the sides of

the test tube. An appearance of reddish brown ring at the junction of two layers and the upper layer becomes bluish green which shows the presence of steroids.

2. Test for terpenoids

Noller's test: The substance was warmed with tin and thionyl chloride. A pink coloration indicates the presence of triterpenoids.

3. Test for flavones

a. **Shinoda's test:** To the substance in alcohol, a few magnesium turnings and a few drops of concentrated hydrochloric acid were added and boiled for five minutes. A red coloration indicates the presence of flavones.

b. To the substance in alcohol, 10 percent sodium hydroxide solution or ammonia was added. A dark yellow color indicates the presence of flavones.

4. Test for anthraquinones

Borntrager's test: The substance was macerated with ether and after filtration; aqueous ammonia or caustic soda was added. A pink, red or violet colour in the aqueous layer after shaking indicates the presence of anthraquinones. If present as a glycoside, then the test should be modified by hydrolyzing with hydrochloric acid as the first step.

5. Test for glycosides

The substance was mixed with a little anthrone on a watch glass. One drop of concentrated sulphuric acid was added, made into a paste and warmed gently over a water bath. A dark green coloration indicates the presence of glycosides.

6. Test for sugars

The substance was mixed with Fehling's I and II solutions. A red coloration indicates the presence of sugars.

7. Test for alkaloids

a. To the test substance, a few drops of acetic acid and Dragendorff's reagent were added and shaken well. An orange red precipitate indicates the presence of alkaloids.

b. The substance was mixed with little amount of dilute hydrochloric acid and Mayer's reagent. A white or cream precipitate indicates the presence of alkaloids.

8. Test for quinones

To the test substance, sodium hydroxide was added. A blue green or red colour indicates the presence of quinones.

9. Test for phenols

To the substance, a few drops of alcohol and ferric chloride solution were added. A bluish green or red color indicates the presence of phenol.

10. Test for tannins

The substance was mixed with basic lead acetate solution. A white precipitate indicates the presence of tannins.

11. Test for saponins

The substance was shaken with water. A copious lather formation indicates the presence of saponins.

12. Test for proteins and free amino acids

a. The substance was treated with sulphosalicylic acid solution. A white precipitate indicates the presence of proteins.

b. The substance was mixed with Millon's reagent and heated on a water bath. A red precipitate indicated the presence of proteins.

The results obtained for the qualitative chemical tests are presented in **table 6.**

RESULTS AND DISCUSSION

Table 6: Preliminary phytochemical screening for various crude extracts of *T.fragrans*

S. No.	Chemical Test	Petroleum ether extract	Ether extract	Ethyl acetate extract	Methanol extract	Ethanol extract	Aqueous extract
1.	Terpenoids	-	-	-	-	-	-
2.	Flavonoids	-	-	-	+	+	+
3.	Steroids	+	+	+	-	-	-
4.	Anthraquinone	-	-	-	-	-	-
5.	Glycosides	-	-	-	-	-	-
6.	Sugars	-	-	-	+	+	+
7.	Alkaloids	-	-	-	-	-	-
8.	Quinones	-	-	-	-	-	-
9.	Phenols	-	-	-	+	+	+
10.	Tannins	-	-	-	+	+	+
11.	Saponins	-	-	-	-	-	-
12.	Proteins & free amino acids	-	-	-	+	+	+

Note: (+) Present (-) Absent

From the **table 6** it can be seen that the petroleum ether, ether, ethylacetate extract showed the presence of steroids only whereas the ethanolic, methanolic and aqueous extracts showed the presence of sugar, phenols, flavonoids, tannins and proteins.

The preliminary phytochemical screening revealed the presence of some phytoconstituents in both ethanolic and methanolic extracts and the extractive value for methanol was more. Hence this menstrum was used for extraction of the powder material. The methanolic extract was used for the quantitative estimation of phytoconstituuents, TLC and HPTLC fingerprints and pharmacological screening.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

A particular group of compound present in the crude extracts can be quantified by means of using standard or reference marker compound and then reporting them as equivalent to that much amount of compound present in that extract as per standard compound.

Determination of total flavanoid and phenol content [42-46]

Principle

The aluminum chloride colorimetric technique was used for estimation of total flavonoid estimation [45]. Aluminum ions form stable complexes with C4 keto group and either to C3 or C5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavonoids [45]. These complexes showed a strong absorption at 415nm which is used for the estimation of flavonoids.

The total phenol content of *Thunbergia fragrans Roxb*. was determined by the Folin-Ciocalteu colorimetric method [43]. The Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. The method measures the amount of substance needed to inhibit the oxidation of the reagent [47].

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% aluminum chloride

1M potassium acetate

10% sodium carbonate

1N Folin-Ciocalteu reagent

Procedure for flavanoid content

An aliquot quantity of quercetin [48] was dissolved in ethanol to get a stock solution of 1mg/mL. Further dilutions were made to get concentrations ranging from 20-100µg/mL. 1ml of the above standard solutions were taken in different volumetric flasks, 0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and the final volume was then made up to 5mL with distilled water. After 20min the absorbance was measured at 415nm. A sample without aluminium chloride was used as a blank. From the absorbance obtained, a calibration curve was constructed by plotting concentration versus absorbance of quercetin **(Fig.11).** 1mL of methanolic extract at concentrations 40µg/mL and 80µg/mL were taken and the reaction was carried out as above and the absorbance was measured at 415nm after 20min and the readings were tabulated in **Table 7.** The amount of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg querecetin equivalents /g of extract.

Procedure for phenol content

Gallic was used as a standard; 1mg/mL stock solution was prepared that working dilutions made from 2-10µg/mL. To these solutions 0.5mL of Folin-Ciocalteu reagent and 0.5mL of sodium carbonate was added and the final volume was made upto 10mL with distilled water. The absorbance was measured at 760nm after incubation at room temperature for 30min. The methanolic extract (0.5mL of 500µg/mL) was mixed with 0.5mL of Folin-Ciocalteu reagent and 0.5mL of 10% sodium carbonate and final volume was made up to 10mL with distilled water, and the absorbance was measured at 760nm after incubation at room temperature for 30min. A calibration curve was constructed by plotting concentration versus absorbance of gallic acid **Fig.12.** A linear regression equation was formed and the amount of phenolic compounds was determined using this equation. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of extract. The results are tabulated in **table 8.**

Results and discussion

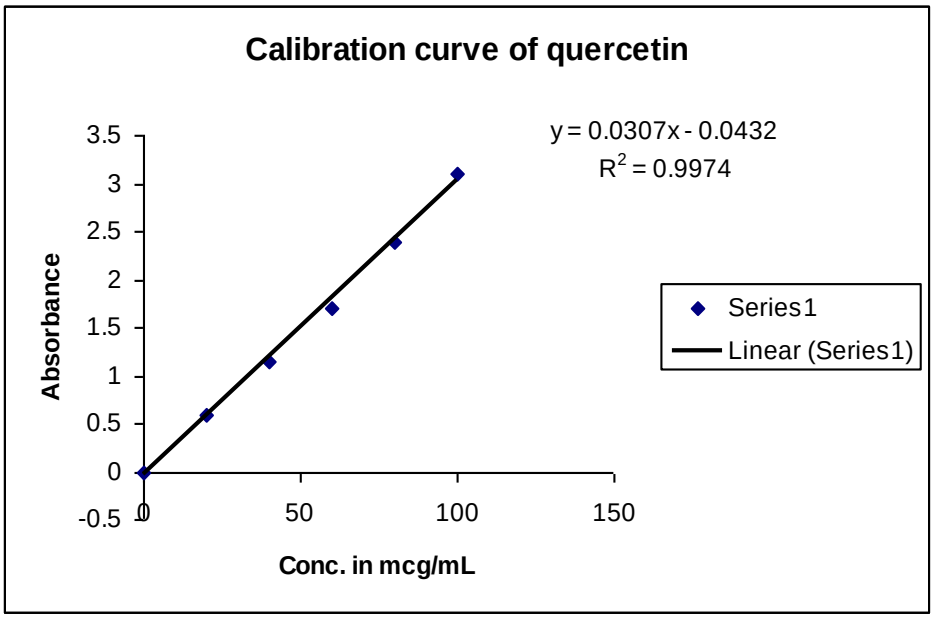
The flavanoid and phenol content for various concentration of methanolic extract was tabulated in **tables 7 & 8.** The linear regression equation was found to be y=0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of flavonoid content present in the methanolic extract of *T.fragrans* in terms mg quercetin equivalent/g of extract was found to be 80.82 ± 4.45mg by using the above linear regression equation.

Table 7: Total flavonoid content per gram of extract in terms of quercetin by aluminium chloride method

S. No.	Conc. of quercetin in µg/mL	Absorbance at 415nm	Conc. of methanolic extract in µg/Ml	Absorbance at 415nm	Amt of total flavonoid content in terms mg quercetin equivalent/ g of extract
1	20	0.589 ± 0.01	40	0.156 ± 0.003	81.24 ± 4.033
2	40	1.151 ± 0.04	80	0.340 ± 0.002	80.40 ± 4.87
3	60	1.710 ± 0.09		**Average**	**80.82 ± 4.45**
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

***mean of three readings** ± SEM

Fig. 11: Calibration curve of quercetin



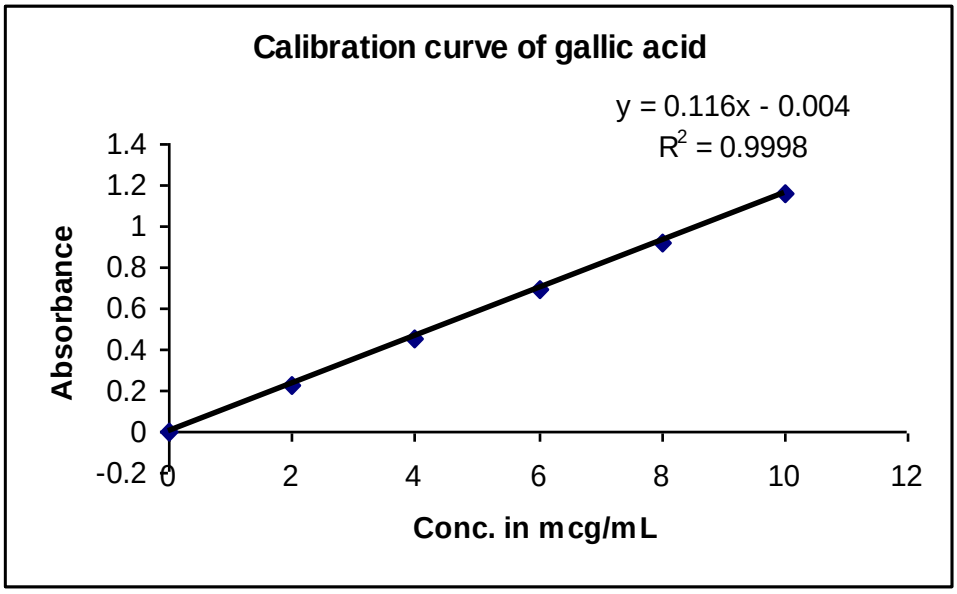
The linear regression equation was found to be y=0.118x-0.004 while the correlation was found to be 0.9998 for gallic acid . The amount of phenolic content present in the extract in terms mg GAE/g of extract was found to be 29.07±0.62 by using the above linear regression equation.

Table 8: Total phenolic content in methanolic extract of *T. fragrans* in terms of gallic acid equivalents

S. No	Conc. of gallic acid in µg/mL	Absorbance at 760nm	Conc. of methanolic extract in µg/mL	Absorbance at 760nm	Amount of total phenolic content in terms mgGAE/g of extract
1	2	0.229 ± 0.010	50	0.168 ± .006	26.81 ± 0.73
2	4	0.452 ± 0.006	100	0.348± 0.018	30.8± 0.45
3	6	0.695 ± 0.005	150	0.470± 0.017	29.60± 0.65
4	8	0.918 ± 0.031		**Average**	**29.07± 0.62**
5	10	1.162 ± 0.028			

***mean of three readings** ± SEM

Fig. 12: Calibration curve of gallic acid



he flavanoid and phenolic content of *T. fragrans* methanolic extract was done using suitable methods with respect to the standards quercetin and gallic acid respectively. Phenols and flavonoids are the group of compounds which inhibit the oxidation reactions. The phenol content was quantified by Folin-Ciocalteu reagent and it was found to be 29.07±0.62 mg of GAE/g of extract of *T.fragrans*. The flavanoid content was found to be

80.82±4.45 mg of quercetin equivalent /g of methanolic extract of *T. fragrans*. Flavanoid content was found to be nearly three fold higher than that compared to phenol content of the extract.

CHROMATOGRAPHY

Chromatography is a technique by which molecular mixtures can be separated according to the relative affinity of solutes between two immiscible phases. One of the phase is fixed of large surface area called stationary phase, while other moves through or over the surface of the fixed phase called mobile phase. Chromatographic methods can be classified according to the nature of the stationary and mobile phases. If the stationary phase is a solid, the process is called as adsorption chromatography and if the stationary phase is a liquid, it is termed as partition chromatography.

The various types of chromatography include paper chromatography (PC), thin layer chromatography (TLC), column chromatography (CC), gas chromatography (GC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC).

Thin Layer Chromatography [41]

Thin layer chromatography (TLC) is a simple technique to adopt for separation and identification of organic compounds. Here the principle involved is adsorption, where the solid stationary phase is used. Depending upon the distribution coefficients, the compounds are distributed on the surface of the adsorbent. The compound, which is readily soluble but not strongly adsorbed, moves up along with the solvent and that not so soluble but more strongly adsorbed move up less readily leading to the separation of compounds.

Materials and methods

Preparation of TLC Plates

Silica gel G was made as a slurry in water (1 : 2). Dry, clean glass plates (20cm x 5cm) were laid in a row as a template and silica gel was spared evenly by Stahl's spreader to get uniform thickness (0.25mm). These plates were left on the template for air drying until the transparency of the layer disappeared and dried at 110^{o}C for 30mins and kept in a dessicator. The plates were used when required.

Sample Application

The methanolic extract of *T. fragrans* was dissolved in distilled water to get 5mg/mL concentration. Capillary tube was used to make a spot on the TLC plate.

Development of chromatogram

The spot was allowed to dry then the plates developed in a chromatographic tank with hexane: ethy acatete: glacial acetic acid (7:3:0.1). After two third of the plate was developed the plates were taken outside and dried. Plate was observed under UV light at 254 and 366nm.

The R_f values were calculated using the formula R_f = Distance traveled by solute/ Distance traveled by solvent. The results are presented in **table 9.**

High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) is a modern adaptation of TLC with improved versatility, separation efficiency and detection limits. It is used a quality control tool since comparison of chromatograms of different lots can demonstrate the similarities and differences between the test samples and the standard chemical markers. HPTLC is useful for identification of plants and their extracts because each plant species produces a distinct chromatogram, with unique marker compounds used for plant identification. HPTLC is a reliable method for quantitation of nanogram level even when present in complex formulation. HPTLC finger print analysis is used for rapid identity check, for monitoring purity of drugs, for detection of adulterants, for determining whether a material is derived from a defined botanical species and also to known whether the constituents are clearly characterized [49].

HPTLC PROFILE:

Instrument used : CAMAG make HPTLC.

Software : winCATS 1.4.3

Sample Applicator : Linomat 5.

Detection : @254nm & @366nm in Densitometry TLC Scanner 3

Sample preparation : The sample was prepared in corresponding solvents

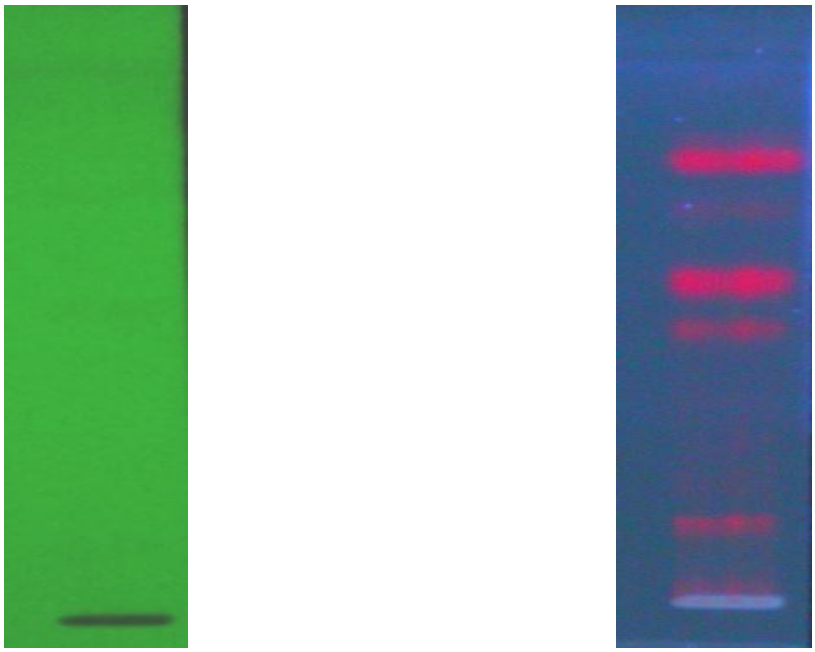
Stationary Phase : HPTLC plates silica gel 60 F 254.

Mobile Phase **:** Toluene: Ethyl acetate: Methanol (7:2:1)

The visualization of the TLC plate of methanolic extract of *T. fragrans* at 254nm and 366nm is presented in **Fig.13 & 14.** The photo of plate at 254nm shows no spots while at 366nm shows the presence 8 spots.

Fig. 13. Visualization at 254nm and 366nm

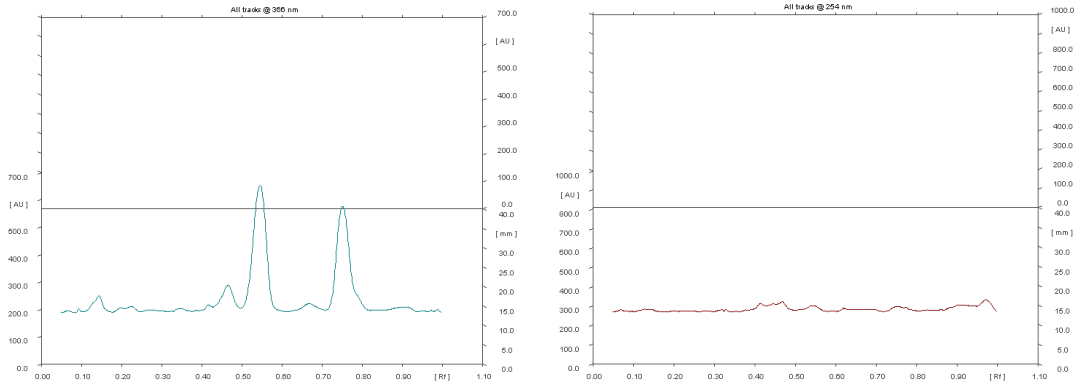
@ 254nm **@366nm**



FINGER PRINTING:

Fig. 14. 3D Display of the fingerprint profile at 254nm and 366nm

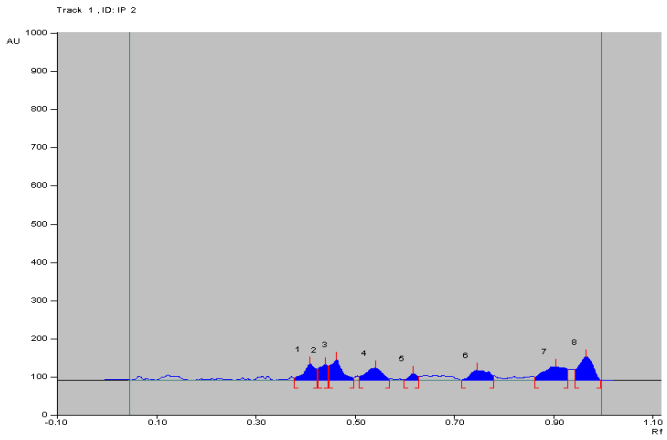
254nm **366nm**



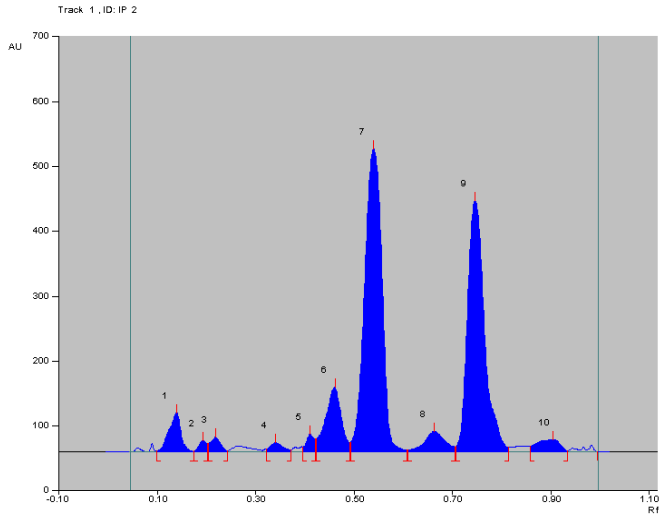
The 3D display of the fingerprint profile and the peak display of methanolic extract of *T.fragrans* at 254nm and 366nm is presented in **Fig.15.** The display at 254nm shows the presence of 8 peaks while at 366nm shows the presence 10 peaks. The R_f values of the peaks along with the area under the curve for each peak at 254 and 366 nm are tabulated in **Table 10.**

Fig. 15. Peak display of methanolic extract of *T. fragrans* at 254nm and 366nm

254nm



366nm



Results and discussion

TLC

The TLC plates showed the presence of different active principles in the methanolic extract of *T.fragrans.* In visible light it shows 7 spots were seen under UV at 354 one spot with the Rf value of 0.95, was taken at 254 nm, it showed four distinct spots. Rf value and color of spots was given in the **table 9.**

Table 9: TLC finger profile of the methanolic extract of *T.fragrans*

Solvent system	Observation	No of spots	Color of spots	R_f values
Haxane: ethyl acetate : glacial acetic acid (7 : 3 : o.1)	366 nm	**1**	Fluorescence	0.95
	254nm	**4**		
		1	Yellowish	0.95
		2	Bluish gray	0.61
		3	Bluish gray	0.53
		4	Light green	0.28
	Visible light	1	Yellow	0.95
		2	Blue	0.61
		3	Blue	0.53
		4	Light green	0.47
		5	Green	0.28
		6	yellow	0.25
		7	Light green	0.21

HPTLC

The HPTLC finger profile showed 8 active constituents at 254 nm and 10 active principles at 366 nm. The 3D display of fingerprint profile shows 8 distinct peaks at 254 nm and 10 individual peaks at 366 nm. The R_f value of the peaks and area under the peaks

was given in the **table 10.** The area under the peak represents the relative amount of active constituents which is responsible for every peak.

Table 10: R_f values and area under the curve for each peak at 254 and 366nm

Peak	@ 254nm		@366nm	
	Rf value	**Area (AU)**	**Rf value**	**Area (AU)**
1	0.41	940.2	0.14	1365.9
2	0.45	656.1	0.20	262.1
3	0.47	1175.9	0.22	426.1
4	0.55	981.6	0.35	339.4
5	0.62	260.6	0.41	430.4
6	0.75	905.1	0.47	2971.4
7	0.91	1479.7	0.54	15417.3
8	0.97	1627.6	0.67	1237.3
9			0.75	12557.2
10			0.91	844.6

TLC and HPTLC finger profile

The methanolic extract was used for in vitro and in vivo pharmacological experiments. Hence the extract was subjected to TLC and HPTLC analysis, which can be utilized for authentication of the plant and its extract.

The TLC plates showed the presence of numerous active principles in the methanolic extract of *T .fragrans.* Since the first showed flouresence color it may be a phenolic compound.

The HPTLC finger print profile of the plant extract showed various bioactive compounds which may be responsible for the studied pharmacological activities. The finger print profile can be utilized for the quality control for different test samples by comparing different chromatograms.

PHARMACOLOGICAL EVALUATION

Systematic pharmacological screening is the hallmark of a robust study. It is therefore imperative to explore every possible biological activity of the plant. The methanolic extract of *T. fragrans* was screened for *in vitro* antidiabetic activity, larvicidal activity, *in vitro* membrane stabilization assay, antibacterial activity, antioxidant activity, DNA damage protection study and anticancer activity in uterine cancer cells.

IN VITRO ANTI DIABETIC ACTIVITY [50-54]

Many plants are good anti diabetic agents and more than 400 plants have been reported for anti diabetic activity. Jebkins *et al.,* proposed the use of plant derived products with high dietary fiber and complex for polysaccharides for diabetic management. Guar gum also has decreased post prandial blood glucose concentrations in several experiments. However, guar gum use has been limited due to its highly viscous nature and unpalatable taste [50].

Control of plasma glucose is the essential factor in diabetes treatment and to decrease the incidence and severity of long-term diabetic complications. The present drug therapies have many limitations in their use. Hence a study about the prevention of glucose movement across the dialysis membrane into external solute was carried out. It is a convenient model for assessing the factors affecting glucose absorption *in vitro.* Instead of conventional animal model, we used dialysis membrane to study the glucose absorption into blood which insists the blood glucose management without stimulation of insulin and pharmacological limitations of conventional drug therapies [51, 54].

Materials and methods

Chemicals required

1.65mM D-glucose

0.15M NaCl

Dialysis membrane (Hi Media 150)

Instruments required

UV Visible spectrophotometer Shimadzu 1750PC

Magnetic stirrer

Procedure [55]

The method described by Gallagher *et al.,* was modified and adopted for evaluation of antidiabetic activity through inhibition of glucose movement *in vitro.* This *in vitro* model consists of dialysis membrance (Hi Media 150) into which 2mL of 1.65mM D-glucose in 0.15M NaCl and 1mL of various concentration of methanolic extract of *T. fragrans* in distilled water were added. The membrane was tied at both ands tightly; precaution was taken to avoid the loss of contents during tying. The membrane was placed in a beaker with 100mL of 0.15M NaCl beaker placed on magnetic stirrer and kept at a temperature (20 ± 2˚C) **Fig. 16**. The movement of glucose into external solution was monitored by removing a sample every one hour for hours as illustrated. Glucose control (without plant extract), extract blank (without glucose) also were done as per procedure mentioned above. The released glucose was quantified using glucose oxidase method of analysis (GOD/POD Kit). The results are expressed as in mean ± SEM in mM concentration of glucose and are reported in the **table 11 and presented in Figs. 17 and 18.**

Fig.16: *In vitro* antidiabetic activity using dialysis membrane

Results and discussion

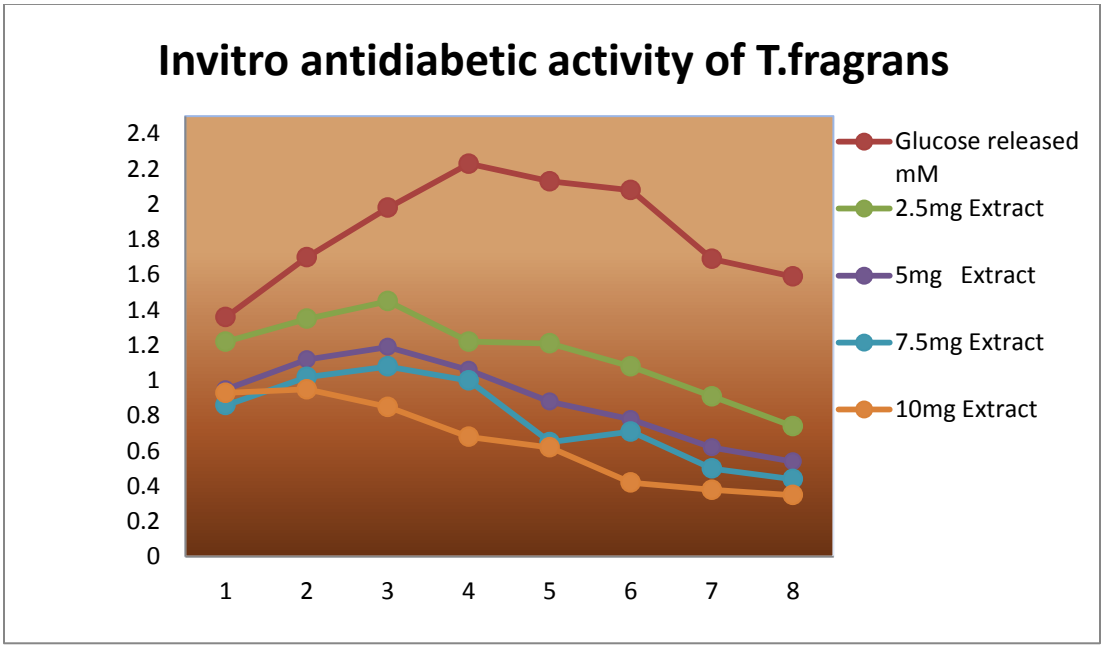
The results obtained for *in vitro* anti diabetic activity is presented in **table 11** and the pictorial representations are presented in **Fig. 17.**

Table 11: *In vitro* anti diabetic effect of *T. fragrans*

Hour	Glucose released mM	2.5mg Extract	5mg Extract	7.5mg Extract	10mg Extract
1	1.36±0.03	1.22±0.06	0.95±0.008	0.86 ± 0.04	0.93 ±0.06
2	1.79±0.07	1.35±0.06	1.12±0.04	1.02± 0.01	0.95± 0.09
3	1.98±0.06	1.45±0.04	1.19±0.04	1.08± 0.05	0.85± 0.05
4	2.23±0.07	1.22±0.08	1.06±0.01	1± 0.05	0.68± 0.02
5	2.13±0.06	1.21±0.06	0.88±0.09	0.65± 0.09	0.62± 0.04
6	2.08±0.07	1.08±0.06	0.78±0.01	0.71± 0.01	0.42± 0.05
7	1.69±0.05	0.91±0.02	0.62±0.06	0.5± 0.04	0.38± 0.07
8	1.59±0.02	0.74±0.05	0.54±0.02	0.44± 0.05	0.35±0.01

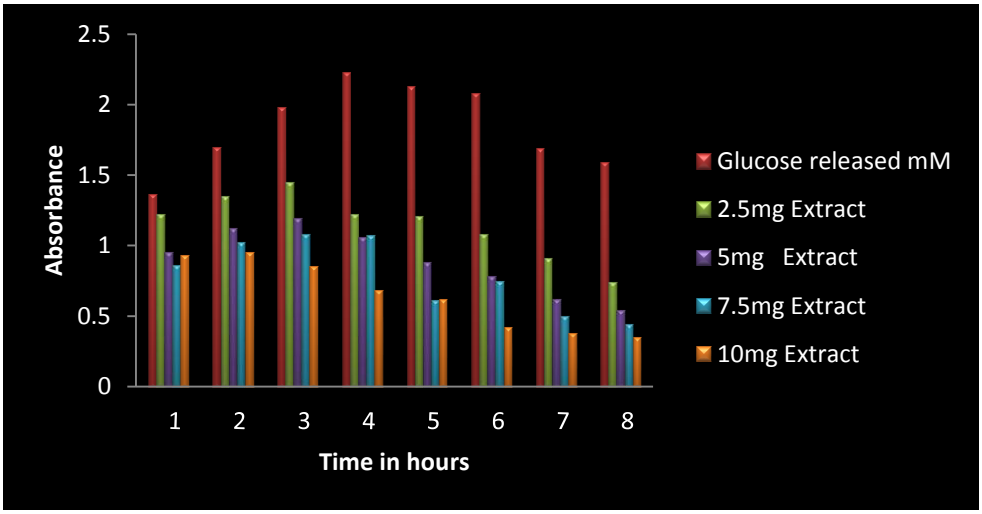
***Mean of three readings±SEM**

Fig. 17: *In vitro* anti diabetic effect of various concentrations of methanolic extract of *T. fragrans*



In this study methanolic extract of *T .fragrans* showed inhibition of glucose movement when compared to glucose control. A dose dependent glucose release inhibition was observed with respect to control.

Fig. 18 : Graphical representation of invitro glucose release study



A maximum inhibition of glucose absorption was observed with 10mg/mL extract which was up to 77%. In case of 7.5mg/mL concentration a 72 percent of inhibition observed. There is deviation of 7.5 mg/mL extract at 5^{th} h, this may be due to the negative speed control in magnetic stirrer.

Diabetes mellitus has already emerged as an alarming disease in the world, and has limitations for current drug therapies; there is an urgent need for search of more efficacious drugs with no or minimum side effects. In the treatment of *Diabetes mellitus*, blood glucose management is an important parameter for the treatment of diabetes. This control can be achieved by three ways [56].

1. Stimulating insulin/ function of beta cells.

2. Decreasing glucose synthesis through alteration in the activities of glucose -6-phosphate and fructose 1,6 bis phosphate.

3. Inhibiting the absorption/transport of glucose.

Among these ways, preventing the excess of glucose across intestinal epithelial cells has a fundamental importance. Here the study was carried out through dialysis membrane which is biologically equal to intestine. *T. fragrans* has positive inhibiting glucose absorption action and in a dose dependant manner. The flavonoid content in the extract may contribute to this glucose inhibition on dialysis membrane. Several studies were reported on the effect of flavanoid on absorption of glucose. Flavonoids have promising antidiabetic effect in various models and since the methanolic extract of *T. fragrans* has high flavanoid content, this may be responsible of *in vitro* antidiabetic activity [57, 58, 59]. This study has highlighted the antidiabetic activity and mechanism of action

of *T. fragrans* also. Further studies need to be conducted in order to confirm the *in vivo* action of *T. fragrans*.

LARVICIDAL ACTIVITY OF *T. FRAGRANS*

Mosquitoes transmit many serious human diseases like malaria, filariasis, Japanese encephalitis, dengue hemorrhagic fever, chikungunya and yellow fever which cause millions of deaths every year. The mosquitoes need to be controlled. Chemical insecticides being used today has many limitations like physiological resistance to vectors and adverse environmental effects etc., and hence plant products are one of the best alternatives for mosquito control [61, 62].

Procedure [62]

Larvicidal bioassay was performed on late 3^{rd} and early 4^{th} instar larvae of *Anopheles stephensi,* a primary vector of urban malaria, *Culex quiquefaciatus*, a common vector of filariasis, *Aedes aegypti*, common vector of dengue and yellow fever and *Toxorhynchites splenders*. The larvae were obtained from ICMR, Madurai. Twenty larvae were released in 500mL beaker containing 200mL distilled water with varying concentration of plant extract (1, 10, 50 & 100mg) the larvae were provided a dog biscuit and yeast powder in a 3:2 ratio as nutrients. The experiments were carried out at room temperature (26^0C± 2^0 C). Three replicates of each concentration were run under the same microclimatic conditions along with untreated control. Mortality was monitored for 24 hours.

Results & discussion

The results obtained for the larvicidal effect of metanolic extract of *T. fragrans* are presented in **table 12.**

Table.12: Evaluation of larvicidal effect of *T. fragrans*

S. No	Mosquito species	Methanolic extract of *T.fragrans*			
		1mg/mL	10mg/mL	50mg/mL	100mg/mL
1	*Anopheles stephensi*	_	_	_	_
2	*Culex quiquefaciatus*	_	_	_	_
3	*Aedes aegypti*	_	_	_	_
4	*Toxorhynchites splenders*	_	_	_	_

[+] indicates positive larvicidal effect, **[-]** indicates negative larvicidal effect

No mortality was observed in 24 hours. In the study no mortality was observed in 24 hours. The extract did not demonstrate any significant larvicidal effects on mosquitoe larvae. In contrast, the larvae are viable even after 48hours. Hence, *T. fragrans* methonalic extract does not have larvicidal activity.

DNA DAMAGE PROTECTION STUDY [62-65]

Flavonoids are the compounds which have the ability to protect against the damaging action of free radicals. Free radicals have been proved to interfere with the propagation reaction and formation of free radicals either by chelating the transition metal or by inhibiting the enzyme involved in the initiation reaction. Reactive endogenous species involved in the etiology of diverse human diseases, such as inflammation, coronary heart diseases, neuro degenerative disease and cancer. Since DNA damage has been particularly implicated in carcinogenesis, this study was carried out to investigate the effect of *T. fragrans* methanolic extract on DNA cleavage.

Materials and methods [66]

Reagents

Alkaline lysis solution I

Alkaline lysis solution II

Alkaline lysis solution III

Antibiotic for plasmid selection-Ampicillin 15 µg/ml

Ethanol and isopropanol

STE(Saline Tris EDTA)

TE (Tris EDTA)

Lysozyme 10mg/mL

10% Agarose gel

Luria-Bertani media

Instruments

Refrigerated centrifuge, [Eppendorf]

Micropipette

Centrifuge tubes

Orbital shaker

Procedure

Preparation of media [66]

2 g of Luria-Bertani media was weighed accurately and transferred to reagent bottle with 100mL of distilled water. Amphicillin was added to the media and autoclaved and used for culture of *E coli* strain.

Preparation of alkaline lysis solution I [66]

Alkaline lysis solution I was prepared in such a way that it contains 50mM D-glucose, 25mM of Tris-HCl and 10mM EDTA and autoclaved for 15min at 15psi ($1.05kg/cm^2$) on liquid cycle and stored at 4^oC.

Preparation of alkaline lysis solution II

0.2N sodium hydroxide (freshly prepared), 1% w/v sodium lauryl sulfate (SDS). The solution was prepared freshly and used at room temperature.

Preparation of alkaline lysis solution III

5M potassium acetate, glacial acetic acid and water were mixed together. The resulting solution is 3M with respect to potassium and 5M with respect to acetate. The solution was stored at 4^{o}C then used.

STE

10mM Tris-Cl (pH 8), 0.1M NaCl and 1mM EDTA (pH 8) were mixed together. It was sterilized by autoclaving for 10mins at 15psi and stored at 4^{o}C.

TE

100mM Tris-Cl, 10mM EDTA (pH 8) were mixed together. The solution was sterilized by autoclaving for 20mins at 15psi ($1.05kg/cm^2$) on liquid cycle and stored at room temperature.

Lysozyme Preparation

Solid lysozyme was dissolved at the concentration of 10mg/mL in Tris-HCl immediately before use (Lysozyme will not work efficiently if the solution is less than pH 8).

Preparation of Cell culture [66]

PBr322 *E.coli* strain was inoculated in 50mL of LB medium containing Ampicillin 15µg/mL. It was incubated at room temperature with vigorous shaking of 300 cycles/min on rotator shaker until the bacterial rich late log phase [OD_{600}= 0.6] was obtained. The bacterial cells were harvested from the culture by centrifugation at 4^{o}C, 4000 rpm for

15min. Supernatant liquid was discarded, bacterial cells collected and they were re-suspended in frozen (ice cold) STE and centrifuged at 4^{o}C, 4000 rpm for 15mins.

Isolation of plasmid DNA

The bacterial pellet obtained was re-suspended in 1.8mL of alkaline lysis solution I and freshly prepared 0.2mL of 10mg/mL lysozyme was added. To this solution 4mL of freshly prepared alkaline lysis solution II was added and mixed gently by inverting the centrifuge tube several times. It was incubated at room temperature for 5-10mins. To the above solution 2mL of ice cold alkaline lysis solution III was added and the contents were mixed by swirling the centrifuge tube several times.

Bacterial lysate was centrifuged at 7000rpm for 40 mins at 4^{o}C using refrigerated centrifuge and the supernatant liquid was collected using Pasteur pipette. The volume of supernatant liquid was measured transferred to 6 volumes of iso-propanol in fresh centrifuge tubes. The content were mixed well and stored at room temperature for 10min and centrifuged at 7000rpm for 30min at room temperature.

The supernatant liquid was removed carefully and the centrifuge tube was inverted on a pad of paper towel to allow the last drops of supernatant to drain away. The pellet was collected and rinsed with 70% ethanol, using Pasteur pipette to remove any beads of liquid that adhere to the walls of the tube. The open centrifuge tube was inverted on the pad of paper towel for few minutes at room temperature to allow the ethanol to evaporate and 1mL of TE (pH 8) added and stored at 4^{o}C.

Agarose slab gel preparation [67]

Agarose weighed about 0.4 grams and transferred to beaker containing 40 mL 1X TBE. It was heated in a microwave until agarose is completely dissolved to get 10%

agarose gel and allowed to cool for 50-55^{o}C for 10 mins. Gel casting system tray was prepared by sealing of gel chamber with tape. 8 well comb was placed in the tray. 2 µl of ethidium bromide was added to cooled gel and poured into gel tray. Then it is allowed to cool for 15-30 mins at room temperature. Comb was removed and the gel placed in electrophoresis chamber and covered with buffer.

DNA damage protection study [62]

The experiment was performed in a volume of 20µl of plasmid DNA in TE (pH 8) and 15µl of varying concentrations of *T. fragrans* methanolic extract (25µg, 50µg, 60µg, 70µg). 1µl of 2.5mM of H_2O_2 was added just before irradiation of samples with UV light. Samples in polyethylene centrifuge tubes were placed directly on the surface of UV-trans illuminator at 300nm. The samples were irradiated for 10min at room temperature. After irradiation 3µl of sample with 3µl of gel loading dye were added and they were analyzed by electrophoresis on a 10% agarose horizontal slab gel in Tris-borate buffer (45mM/L Tris borate, 1mM/L EDTA). Untreated plasmid DNA, plasmid DNA with UV irradiation, plasmid DNA with H_2O_2 and plasmid DNA with both UV and H_2O_2 were also run on gel electrophoresis at 100 V for 1h. Gel was stained with 2µl ethidium bromide during gel preparation. Gel was scanned on gel documentation system (Gel Dox-XR, BioRad) and represented in **Fig.19-22.**

Fig 19: Agarose gel casting

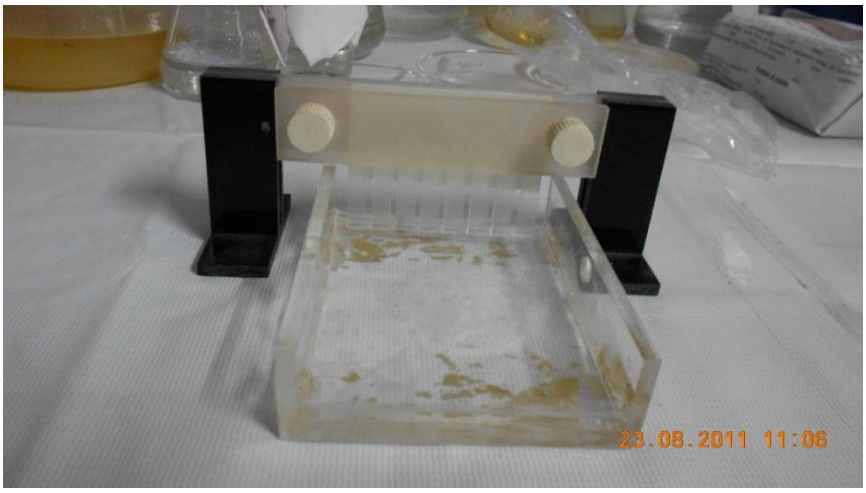


Fig. 20: Sample application on Gel electrophoresis apparatus

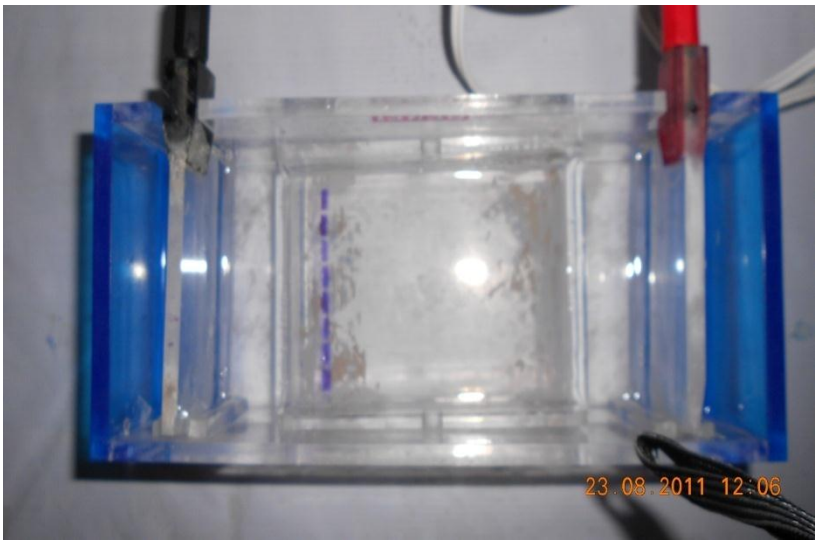


Fig.21: Sample movement on Gel electrophoresis

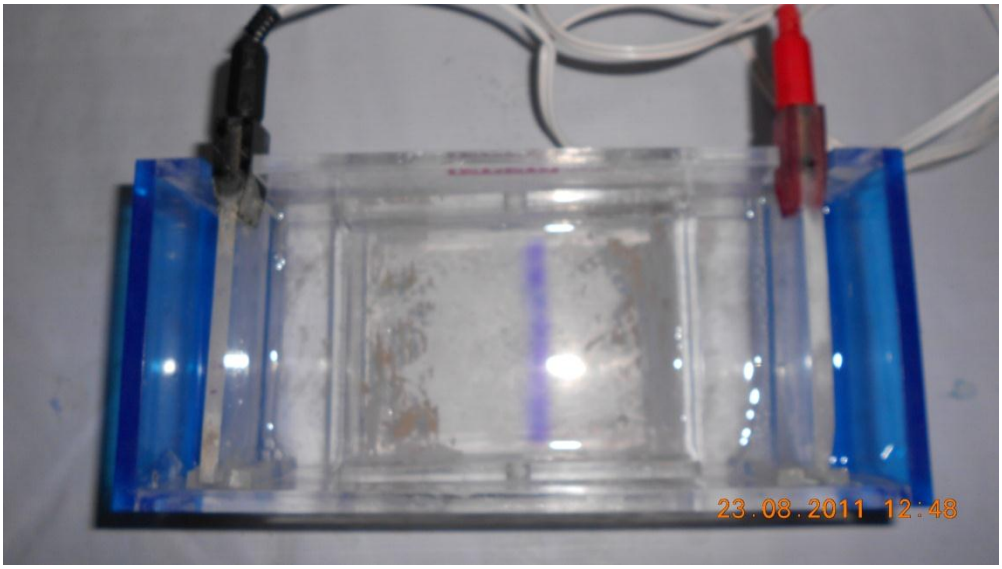
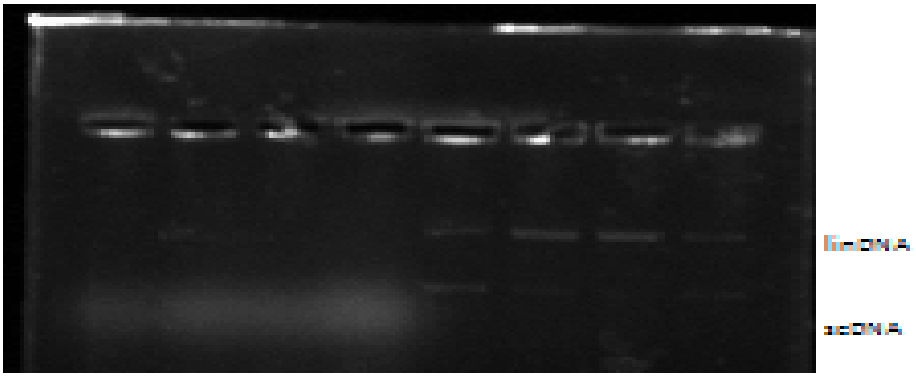


Fig. 22: Electrophoretic pattern of DNA



Lane 1- control DNA;Lane 2- Control DNA - UV Treated; Lane 3- Control DNA - H_2O_2 Treated;
Lane 4- Control DNA - UV + H_2O_2 Treated; Lane 5- Extract 70µg UV + H_2O_2 Treated;
Lane6- Extract 60µg UV + H_2O_2Treated; Lane7- Extract 50µg UV + H_2O_2 Treated;
Lane 8- Extract 25 µg UV + H_2O_2 Treated

Results and Discussion

The **Fig.22** showed the electrophoretic pattern of DNA after UV photolysis and H_2O_2 in absence and presence of methanolic extracts of aerial parts of *T. fragrans*. The

pBR322 DNA plasmid showed two bands on agarose gel electrophoresis (lane 1), the faster moving band corresponding to the super coiled circular DNA (scDNA) and the slower moving band being the open circular form (ocDNA). In presence of H_2O_2 and UV irradiation (lane 4) DNA shows the cleavage of scDNA to ocDNA and linear form (linDNA), indicating the OH* radical generated from UV photolysis of H_2O_2 produced DNA scission [62].

In the living cells ROS (Reactive oxygen species) are formed continuously by the metabolic reactions. The antioxidant defense systems cannot provide complete protection from ROS. This results in oxidative damage to DNA. In experimental studies in animal and *in virto* models have suggested as an important factor for the carcinogenisis. Modified DNA form abundantly and the damaged nucleosides accumulate with age in mitochondrial and nuclear DNA. Several oxidative modifications of base and sugar residues also occur frequently in DNA [62].

In the present study, the methanolic extract of *T. fragrans* extract prevented the formation of linear and open coiled DNA. It also showed a strong protection against UV and H_2O_2 induced mutations. Further studies are to be carried out to confirm whether this plant has *in vivo* DNA protection effect.

ANTIOXIDANT ACTIVITY [68-70]

Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert the effects by scavenging reactive oxygen species (ROS) or preventing the generation of ROS. Naturally there is balance between the amount of free radicals produced in the body and antioxidants. The amount of antioxidants in normal physiological conditions is insufficient to neutralize free radicals generated in diseases or

ill conditions. Therefore it is obvious to enrich our diet with antioxidants to protect from harmful diseases.

Oxidative stress is defined in general as excess formulation and or complete removal of highly reactive molecules such as reactive oxygen species, singlet oxygen (O_2*), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxide radical (RO_2) & hydroxyl radical (OH), are thought to cause oxidative damage. Oxidative stress further leads to ageing process and degenerative diseases like cancer, inflammation ,cardio vascular and neurodegenerative diseases etc.,

Even though many compounds from synthetic origin like BHT (butylated hydroxyl toluene), BHA (butylated hydroxyl anisole),tertiary butylated hydxyl quinine (TBHQ) and gallic acid are available, they are known to have toxic and carcinogenic effects and have low solubility [68-70] .Due to this strong limitations have been seen on their use, there is necessity to replace with natural antioxidants .Natural antioxidants have strong antioxidants and low cytotoxicities

Plant derived antioxidants such as tannins, liginins, stilbenes, coumarins, quinines, xanthones, phenolic acids, flavones, flavanols, catechins, anthocyanins and polyanthocyanins have strong antioxidant and preventive acting against degenerative diseases due to the redox properties [71, 72]

Many *in vitro* models for evaluating antioxidant activity are available. Some of them are enumerated below

1. Conjugated diene assay
2. DPPH method
3. Superoxide radical scavenging activity

4. Hydroxyl radical scavenging activity
5. Nitric oxide radical inhibition assay
6. Reducing power method
7. Phosphomolybdenum method
8. Peroxy nitrile radical scavenging activity
9. 2,2-azobis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt method
10. N, N dimethyl-p-phenylene diamine dihydrochloride method
11. Oxygen radical absorbance capacity
12. B-carotene linoleate model
13. Xanthine oxidase method
14. Ferric reducing ability of Plasma
15. Total radical trapping antioxidant potential
16. Cytochrome C test
17. Erythrocyte ghost system
18. Thiobarbituric acid assay etc.

METHOD 1: FREE RADICAL SCAVENGING ACTIVITY USING DIPHENYL PICRYL HYDRAZYL (DPPH) FREE RADICAL [73]

Principle:

This method employs diphenyl picryl hydrazyl radical (DPPH). DPPH is a stable free radical with a distinctive ESR signal. It react antioxidants and gives absorbance at 517nm [74]. It is widely accepted that DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the ethanol solution of DPPH, which is purple in colour shows a strong absorption at 517nm. DPPH radicals react

with suitable reducing agents and then electrons become paired off and the solution loses color stochiometrically with the number of electrons taken up [75].

$$\mathbf{DPPH^{\cdot} + AH \rightarrow DPPH\text{-}H + A^{\cdot}}$$

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

0.1mM DPPH in ethanol

Procedure

A stock solution of DPPH was prepared in 100mL of ethanol. To the 1mL of test samples of different concentrations, 4mL of DPPH 0.1mM was added. Control without test compound was prepared in the same manner. In case of blank, DPPH was replaced by ethanol. The reaction was allowed to be completed in the dark for about 30min. Then the absorbance of test mixtures was read at 517nm. Vitamin C was used as standard. The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical. The percentage scavenging was calculated using the formula [(Control-Test)/Control] x 100 and the concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. The results obtained are presented in **Table 13** and **Fig. 23.**

Method 2: Reducing power assay [76-78]

Principle

This is a spectrophotometric method and is based on the principle that as concentration of the reaction mixture increases absorbance increases as showing an

increased antioxidant activity. The assay is based on the reduction of ferric to ferrous to form (potassium ferricyanide to potassium ferrocyanide) by the sample and the subsequent formation of Prussian blue color with ferric chloride. The absorbance of the blue complex is measured at 700nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

1% potassium ferricyanide

10% trichloro acetic acid

0.2M, pH 6.6 phosphate buffer

0.1% ferric chloride

Procedure

T. *fragrans* extract solution 0.1-0.5mL taken and diluted with water to form final concentration 100-500µg/L) and mixed with 0.75mL phosphate buffer and 0.75mL potassium ferricyanide [K3Fe(CN6)], then mixture was incubated at 50^{o}C for 20min. Trichloro acetic acid was added 0.75mL to the mixture, which was then centrifuged at 3000rpm for 10min. 1.5mL of the supernatant solution was mixed with 1.5mL of distilled water and 0.1mL ferric chloride and absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean ± standard error of the mean. The antioxidant activity was expressed as equivalents of Vitamin C (µg/g). The results obtained are presented in Table **14** and **Fig.24.**

Method 3: Determination of scavenging activity against hydrogen peroxide [79]

Principle:

The principle is based the capacity of the extracts to decompose the hydrogen peroxide to water. The radical scavenging activity against hydrogen peroxide of plant extract was determined by using the method of Ruch *et al*.**[79].**

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

6% hydrogen peroxide diluted with water in the ratio of 1:10

0.1M, pH 7.4 phosphate buffer

Procedure

The methanolic extract of *T.fragrans* was dissolved in distilled water to get a stock solution of 1mg/mL. Varying quantities of the stock solution (22.22-177.78µg/mL) were added to 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and then mixed with 0.2mL of hydrogen peroxide solution. After 10min, absorbance of the reaction mixture was measured at 230nm. The reaction mixture without sample was used as blank. Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula. The results are presented in Table 15 and **Fig. 25.**

$$\%\text{inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100.$$

Result and discussion [62] **:**

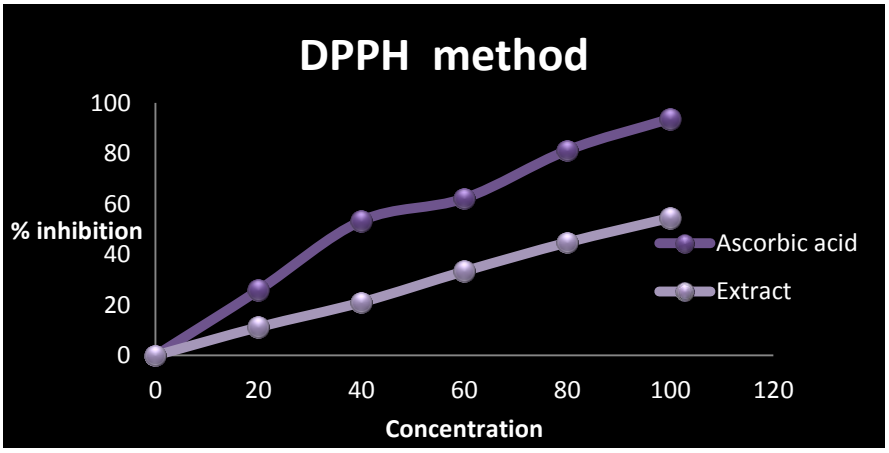
The results obtained on the study of quenching of DPPH free radical at 517nm, reducing power on potassium ferricyanide and scavenging activity against hydrogen peroxide are presented in **tables 13, 14, 15** respectively**.**

Table 13: Percentage inhibition of methanolic extract of *T. fragrans* and standard ascorbic acid against DPPH at 517nm

S. No.	Conc. in µg/mL	Percentage inhibition by standard ascorbic acid	Percentage inhibition by methanolic extract
1	20	25.86 ± 5.63	11.2 ± 1.49
2	40	53.32 ± 4.84	21.05± 1.64
3	60	62.2 ± 7.35	33.36± 2.83
4	80	81.21 ± 5.87	44.75± 2.11
5	100	93.73 ± 0.85	54.49± 2.29
	IC_{50}	**47. 06µg/mL**	**90.35 µg/mL**

***mean of three readings ± SEM**

Fig.23 : DPPH Radical Scavenging Acitivity



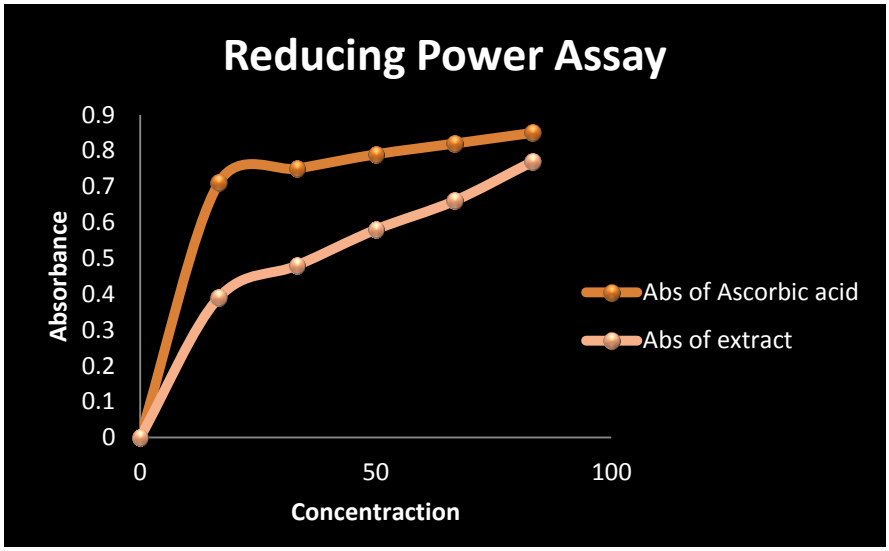
DPPH contain one odd electron and it becomes paired off in presence of a free radical scavenger, which reduce the characteristic absorption of DPPH at 517nm and thus decolorization occurs. This decolorization is stoichiometric with respect to electrons taken up.

Table 14: Reducing power of methanolic extract of *T.fragrans* on potassium ferricyanide

S.No.	Conc. in µg/mL	Abs. of ascorbic acid	Abs. of methanolic extract
1	16.67	0.71±0.03	0.39± 0.02
2	33.33	0.75± 0.03	0.48± 0.02
3	50.00	0.79± 0.01	0.58± 0.03
4	66.67	0.82± 0.01	0.66± 0.03
5	83.33	0.85± 0.003	0.77± 0.04

***mean of three readings ± SEM**

Fig. 24: Reducing Power Assay



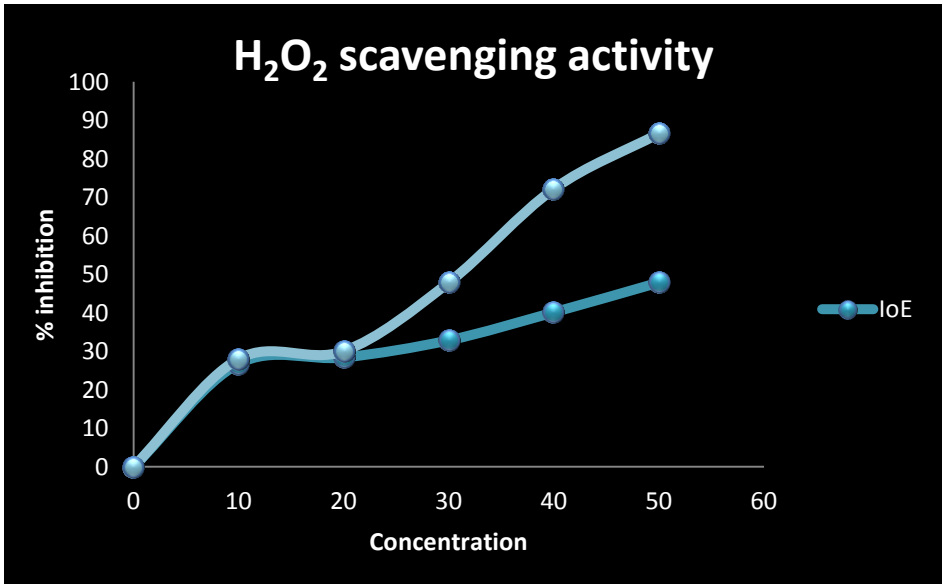
In case of reducing activity of methanolic extract of *T. fragrans*, the transformation of ferric ion (Fe^{3+})to ferrous ion (Fe^{2+}) in the presence of extract was adopted. The highest reducing power was detected for 33µg/mL of extract and the extract exhibited the dose dependent reducing ability.

Table 15: Percentage inhibition of hydrogen peroxide by methanolic extract of *T. fragrans*

S.No.	Conc. in µg/mL	Percentage inhibition	
		Methanolic extract of *T. indicum*	Ascorbic acid
1	10	26.76± 1.32	28.02
2	20	28.39± 2.49	30.12
3	30	32.85± 2.12	47.85
4	40	40.12± 0.97	72.18
5	50	47.87± 2.9	86.56
	IC_{50}	50.48 mcg/mL	28.53 mcg/ML

***mean of three readings ± SEM**

Fig. 25: Percentage inhibition of H_2O_2 by ascorbic acid and methanolic extract of *T. fragrans*



Scavenging activity of the methanolic extract against hydrogen peroxide showed dose dependent action. The methanolic extract of *T. fragrans* showed the maximum percentage inhibition of 47.87% at 50 µg/ml which was comparable to that obtained at 30µg/mL of ascorbic acid.

The methanolic extract of *T. fragrans* exhibited good antioxidant effect with respect to ascorbic acid by reducing power assay, scavenging effect against hydrogen peroxide and free radical scavenging activity against DPPH radical.

ANTICANCER ACTIVITY IN UTERINE CANCER CELLS [80]

Cells of exponential phase of growth were exposed to a cytotoxic drug. The duration of exposure was usually determined as the time required for maximal damage to occur, but was also influenced by the sterility of the drug. After the removal of drug, the cells were allowed to proliferate for two or three population doubling times (PDTs) in order to distinguish viable or non viable cells. Number of surviving cells was determined indirectly by MTT dye reduction assay. In this assay the amount of MTT-formozan produced can be determined spectroscopically, once the MTT-formozan has been dissolved in DMSO.

Materials and method

Sterile:

- Growth medium (MEM- Minimum essential medium)
- Trypsin (0.25% + EDTA, 1mM in PBSA).
- MTT (3 – (4,5 – diamethyl thiazol – 2yl) – 2,5 – diphenyl tetrazolium bromide (Sigma), 50mg/ml filter sterilized)
- Microtitration plates
- Pipette tips preferably in a autoclavable tip box
- Universal containers (or) tubes 30ml – 100ml
- DMSO (Dimethyl sulfoxide)

Instruments:

- Multi channel pipetter

- ELISA Plate analyser, Robonik- read well touch
- Envair electrodyne – Laminar Airflow Hood
- Shel Lab CO_2 incubator.
- Olympus inverted phase contrast microscope CK x 41

Procedure

Human cell lines and its maintenance

Invitro studies with human cell lines were done at Lady Doak College, Madurai. The cells were cultured as per the conditions recommended by the supplier, (NCCS, Pune). Normal human lymphocytes were obtained from the blood of healthy volunteers using density gradient centrifugation with Hi-sep solution (Hi-media). They were cultured in the RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin 20 IU ml^{-1}, streptomycin 15µg ml^{-1} and amphotericin 20 µg ml^{-1}).

Dose response analysis [82-85]

The uterine cancer cells (SiHa) was propagated at 37^0C with 5% CO_2 for 24 h to facilitate attachment in the 25 mm T-flask using CO_2 incubator, the monolayer cells at 80% confluency were trypsinized and seeded on to a 96 well titer plates with a cell suspension of 90µl containing approximately 2 x 10^3 cells per well.

The wells then received 10µL containing dried methanol extract re-suspended in the complete medium at various concentrations of 1.5µg, 3µg, 6µg, 8µg, 16µg, 24µg, 32µg and 40µg. Trypan blue stain (0.4% in PBS) was used to detect the dead and dying tumor cells. The extent of cytotoxicity was measured at 24h using MTT assay [81]. The plant extract treated cells in the micro titer plates were incubated for one hour with 0.025 ml of 5mg mL^{-}

of MTT and then solubilized with 200µL DMSO. The absorbance was measured using a micro plate reader (Bio-Rad model 680, USA.) at 490nm with control wells as blank.

Plates were placed in plastic box and incubated at 37˚C, 5% CO_2 for 2 days starting with 2 and ending with column 11. The cells were observed under microscope for exponential phase of growth.

The plates were taken out and the medium was removed with a pauster pipette then rinsed with PBS (Phosphate saline buffer). PBS was removed and 90µL of medium added to each well. 10µL plant extract with various concentration (1.5, 3, 6, 8, 16, 24 and 32, 40µg) were added to the wells and incubated and at 37˚C , 5% CO_2 for overnight.

After overnight incubation, cells were taken out and medium removed from the cells. 50µL of MTT was added to each well and incubated for 1h 37˚C with 5% CO_2. MTT removed from each well and 200µL of DMSO added to each well. Absorbance was measured at 570nm using Elisa analyzer.

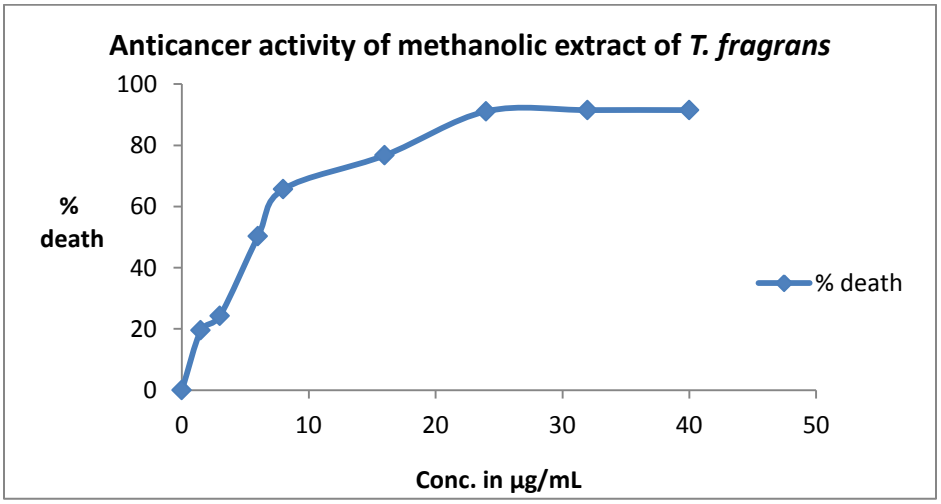
Results and discussion [86-90]

The results for anticancer activity of methanolic extract of *T. fragrans* was represented in the **table 16 and in Fig 26-34.**

Table 16: Anticancer effect of methanolic extract *T. fragrans* on SiHa cells

S.No	Concentration in µg	Absorbance at 570nm	% cell death
1	0	0.507 ± 0.004	0
2	1.5	0.408 ± 0.004	19.53
3	3	0.384 ±0.002	24.26
4	6	0.252 ± 0.001	50.3
5	8	0.174 ± 0.003	65.68
6	16	0.118 ± 0.002	76.72
7	24	0.045 ± 0.002	91.51
8	32	0.043± 0.001	91.51
9	40	0.043 ± 0.001	91.51

Fig.34: Anticancer Activity of methanolic extract of *T. fragrans* against SiHa cells



Uterine cancer is the most prevalent disease in women. Natural compounds have anticancer potential they have been used as a lead compounds for anticancer drugs. The result of the research revealed that the inhibitory effects of *T. fragrans* on the growth of SiHa cells (Uterine cancer cells). The extract showed maximum growth inhibition at 8µg, 16µg, 24µg, 32µg and 40 µg/mL concentration and it is represented in the **table 16**. However, cytopathic effect was observed from lower concentration from 1.5µg/mL itself.

The extract showed a dose dependent inhibitory action on cancer cells and it was represented in the graph **Fig 34.**

The anticancer activity of the methanolic extract may be attributed the presence of flavonoids in it. Flavonoids and phenolic compounds have been proved to inhibit proliferation and angiogenesis of tumor [87, 89].

INVITRO ANTI INFLAMMATORY ACTIVITY OF *T.FRAGRANS* [91-94]

Inflammation is the normal protective response in biological system to tissue injury and it is caused by physical trauma, noxious chemicals or microbiological agents. Irrespective of the type of injury the inflammatory response is suppressed by glucocorticoids. This is the basis of most of their clinical uses. Corticoids are only palliative, do not remove the cause of inflammation: the underlying disease continues to progress while manifestations are dampened. They favour spread of infections as capacity of defensive cells to kill microorganisms is impaired.

Frequent use of corticoids is hazardous. NSAIDs (Non Steroidal Anti-Inflammatory Drugs) are also used to treat inflammation. The major mechanism of action of the NSAIDs is the inhibition of prostaglandin (PG) synthesis or preferential or selective cox-2 inhibition. Inhibition of PG synthesis it may produce toxicities like bleeding, gastric mucosal damage, inhibition of platelet function: delay/prolongation of labour, asthma and anaphylactoid reaction in some individuals. Due to this adverse effects of allopathic drugs there is a need for alternative herbal medicine.

Erythrocytes have been used as a model system by a number of workers for the study of interaction of drugs with membranes [92]. This membrane stabilizing activity of

RBC membrane exhibited by some drugs, serves as a useful in vitro method for assessing the anti-inflammatory activity of various compounds [91]. Here, we studied the membrane stabilizing activity of *T. fragrans.*

Materials and methods

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents required

0.2M sodium phosphate buffer (pH 7.4)

0.36% w/v hyposaline

10%v/v HRBC suspension in isosaline

Procedure [92-94]

The reaction mixture was prepared with 2mL of hyposaline and 1 mL phosphate buffer 0.5mL HRBC suspension in isosaline and varying volumes of T.fragrans methanolic extract (0.1 – 0.5ml) to make final concentration 22 µg, 44 µg, 66 µg, 88 µg and 111 µg/mL . Final volume was made upto 4.5 mL. Control was prepared without extract, extract blank also prepared without HRBC suspension. It was incubated at 50^{o} C for 30 min in hot air oven. Then it was centrifuged at 3000 rpm for 3 min. Supernatent liquid was collected and absorbance was measured at 560 nm. The percentage membrane stabilisation activity of the compounds were determined by the formula.

% membrane stabilization =

[Optical density of control- (Optical density of extract- Optical density of ext blank)] x 100 / Optical density of control .

The results obtained for in vitro membrane stabilization effect is presented in **table 17.**

Table 17. *In vitro* membrane stabilization activity of methanolic extract of T. fragrans

No	T.fragrans Conc. in µg/mL	% inhibition of hemolysis
1	22.22	67.62 ± 2.52
2	44.44	76.02 ± 2.27
3	66.67	81.64± 1.35
4	88.89	84.72 ± 1.38
5	111.11	88.36± 0.96

From the table 17, it can be observed the extract capable of inhibiting the hemolysis 88.36±0.096% . which shows the extract possessed good anti inflammatory activity.

The extrct has good membrane stabilization effect which may be due to flavanoids and phenolic compound.

ANTIBACTERIAL ACTIVITY [95, 96, 97]

The methanolic extract of *T. fragrans* was screened for antibacterial activity on six bacterial strains. Minimum inhibitory concentration was calculated.

Materials and method

MH medium

Plain sterile disc

Petridish

Autoclave

Incubator

Preparation of bacterial cultures:

The various bacterial strains like (*Ecoli, P. aeruginosa, K. Pnemoniae etc.*,) are utilized for screening antibacterial activity. Muller Hinton agar (MH, HI media) was used for culture of bacterial strains. It consists of Beef 2g,casein acid hydrolysate 17.5g,starch 1.5g and agar 17g (pH 7.4 +- 0.2).

Few colonies of the bacterial strains picked from the agar slopes and inoculated in to 4ml peptone water in a test tube. They were incubated for 2-4h to form suspensions. The suspensions was diluted with saline if necessary .

The visual density equivalent to standard prepared by adding 0.5ml of 1% barium chloride to 99.5ml of 1%sulphuric acid. These suspensions were used for seeding.

Disc diffusion technique:

The *T. fragrans* methanol extract was dissolved in DMSO to get concentration of 10mg/ml. The MH media was poured ascetically in to sterilized petridishes and it was swirled to settle agar. The bacterial strains was seeded on the MH agar media by streaking the plate with a sterile swab. The plain sterile discs were impregnated with various volumes of extract and DMSO for negative control and amicasin as positive control was used as standard. The discs were placed on the plate and it was incubated at 37 degree centigrade for 24 hours. The results were read and the zone of inhibition was then measured and they represented in the **tables 18 to 20.**

Results and discussion

Susceptibility tests

The results obtained for the suspecitibily tests are presented in table 18 and the photographs of the same are presented in Fig. 35-40. From the table 18, it can be observed that tested organisms were susceptible at the concentration of 4mg/disc.

Table 18: Susceptibility tests of methanolic extract of *C. guanensis* for various Microorganisms and Fungi

S.No.	Name of the extracts	Conc. of extracts in mg/ disc	1	2	3	4	5	6
1.	Control	---	+	+	+	+	+	+
2.	Std	---	-	-	-	-	-	-
3.	Methanolic extract of *T. fragrans*	2	+	+	+	+	+	+
		4	-	-	-	-	-	-
		6	-	-	-	-	-	-

NOTE:- (+) indicates growth; (-) indicates no growth

1. *Staphylococcus aureus 2. Escherichia coli 3. Klebsiella pneumonia, 4. Proteus mirabilis, 5.Pseudomonas aeruginosa, 6.Candida albicans.*

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was defined as the lowest concentration of the extract that allows no more than 20% growth of microbes after incubation on agar at 37ºC for 18-48h. The minimum inhibitory concentrations against various organisms are presented in table 19.

Table 19: MIC of methanolic extract of *T. fragrans* against various microorganisms

S. No	Name of the organism	Minimum inhibitory concentration (in mg/disc)
1.	*Staphylococcus aureus*	4
2.	*Escherichia coli*	4
3.	*Klebsiella pneumonia*	4
4.	*Proteus mirabilis*	4
5.	*Pseudomonas aeruginosa*	4
6.	*Candida albicans*	4

Zone of inhibition

The results obtained for the antibiotic disc diffusion technique are presented in **Table 20** and **Fig.35-40.** From the **table 20**, it can be observed that the zones of inhibition of the methanolic extract of *T. fragrans* for the tested organisms was less than that produced by the standard Amikacin.

The zone of inhibition obtained against the tested organisms is presented in **table 20 and Fig 35-40.**

Table 20: Antibiotic disc diffusion assay against various microorganisms and fungi

S. No	Name of the organism	Zone of inhibition (in mm)*	
		Standard	Methanolic extract
1.	*Staphylococcus aureus*	21.0 ± 1.0	17.0 ± 0.5
2.	*Escherichia coli*	24.0 ± 0.0	16.0 ± 0.5
3.	*Klebsiella pneumonia*	21.0 ± 3.0	15.0 ± 0.5
4.	*Proteus mirabilis*	26.0 ± 0.0	20.0 ± 0.5
5.	*Pseudomonas aeruginosa*	25.0 ± 0.5	18.0 ± 0.0
6.	*Candida albicans*	26.3 ± 0.2	18.0 ± 0.5

*** mean of 2 readings**

Developing nations face major microbial infections hence research and development on antimicrobial therapeutics from plant origin could be invaluable to face the economical loss due to microbial infections. Plants based antimicrobial agents are high therapeutic potential. The zone of inhibition by the disc diffusion technique showed the antibacterial effect at 4mg/disc. The extract showed effect against *E coli, P. aeruginosa, K. pneumoniae, S. aureuss.* Thus it was concluded that the extract has a wide spectrum of antibacterial activity.

SUMMARY AND CONCLUSION

The plant kingdom holds many species of plant containing medicinally active phytoconstituents. Recently there has been a revival of interest on plants. In this work an attempt was made to explore the plant *T. fragrans* Roxb.

The dissertation is entitled **"Pharmacognostic, phytochemical and pharmacological evaluation of T. fragrans Roxb.(Acanthaceae)''**

Review of literature revealed very little information on the *Thunbergia fragrans* on pharmacological and phytochemical work. Hence detailed pharmacognostical study was carried out including macroscopical, microscopical characterization and quantitative microscope analytical parameters, standardization parameters. The observed macroscopical, microscopical, cytomorphological features has added more weightage to the authenticity of the plant.

TLC and HPTLC fingerprint profile evaluation gives values which are constant and can be utilized for identification for its quality, purity and authentication of the plant *T. fragrans.*

Preliminary phytochemical screening and quantitative estimation of phytoconstituents goives clear idea on the type of secondary metabolites present in the *T. fragrans,* and the quantitation revealed the amount of flavonoids and phenols present in *T. fragrans* methanolic extract.

Pharmacological studies revealed that the methanolic extract of *T. fragrans* has good anti-diabetic activity and this may be attributed to the presence of poly phenolic and flavanoid compounds.

The extract showed good in-vitro anti inflammatory activity which also proves the folklore claim of the plant.

The antibacterial activity showed the broad spectrum of activity against the tested gram –ve and gram +ve bacteria.

The three antioxidant studies showed good antioxidant potential of the plant extract. Detailed studies on *E-coli* plasmid DNA ensures the UV and H_2O_2 protection and anti-mutagenicity of *T. fragrans.*

Since ROS and DNA damage are primary responsible factors for many types of cancer. Anticancer activity was carried out.

Many researchers revealed that diabetes is a primary risk factor for uterine cancer, hence the uterine cancer cells were selected for the anticancer activity. Anticancer study confirms the good anticancer potential of *T. fragrans.*

The antidiabetic activity and anticancer activity may be attributed to the presence of flavanoids and phenolic compounds. It may also be attributed to other pharmacological actions.

The research results revealed good anticancer potential against uterine cancer cells. Further pharmacological research using other cells the anticancer activity must be carried out in order to establish whether this plant can be used as a potential source of anticancer medicine. *T. fragrans* extract has antidiabetic action (inhibiton of glucose) which is a primary risk factor of uterine cancer cells. Further *in vivo* studies need to confirm the anti diabetic and anticancer activity of *T.fragrans*.

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Fig.26 :Control cell lines (monolayer)

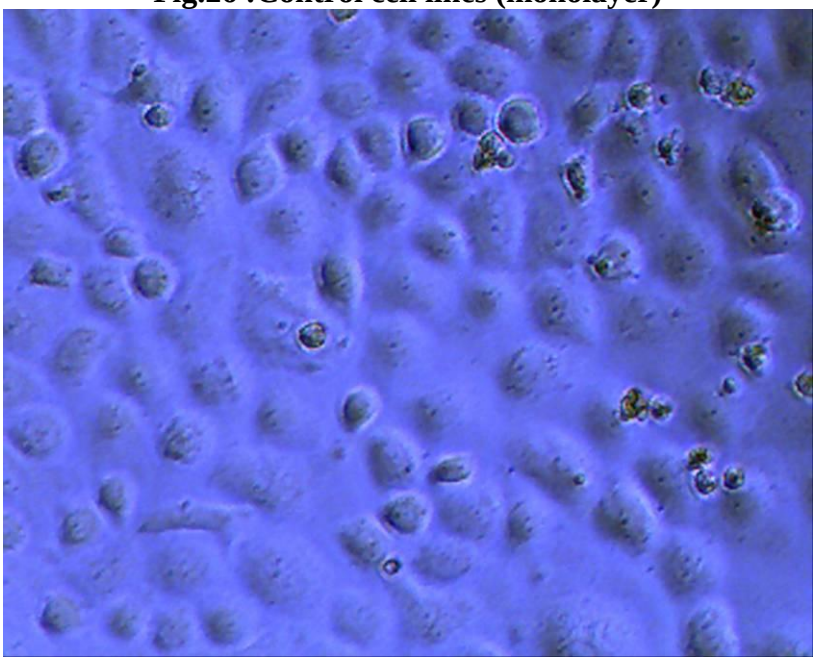

Fig. 27: Cell lines treated with 3 µg extract of *T. frgarans*

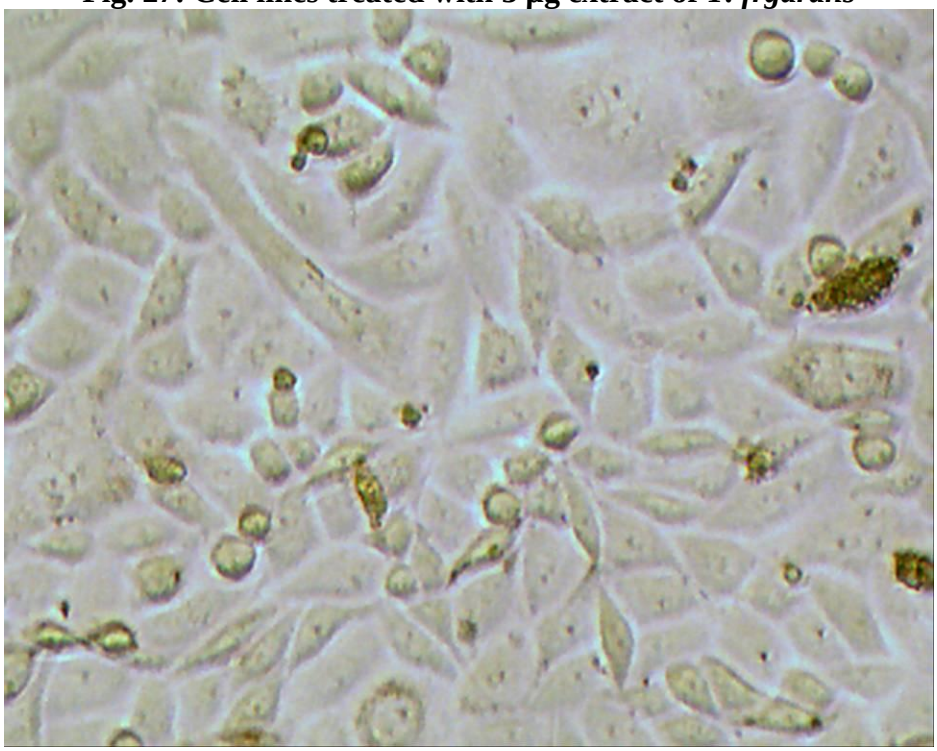

Fig. 28: Cell lines treated with 6 µg extract of *T. frgarans*

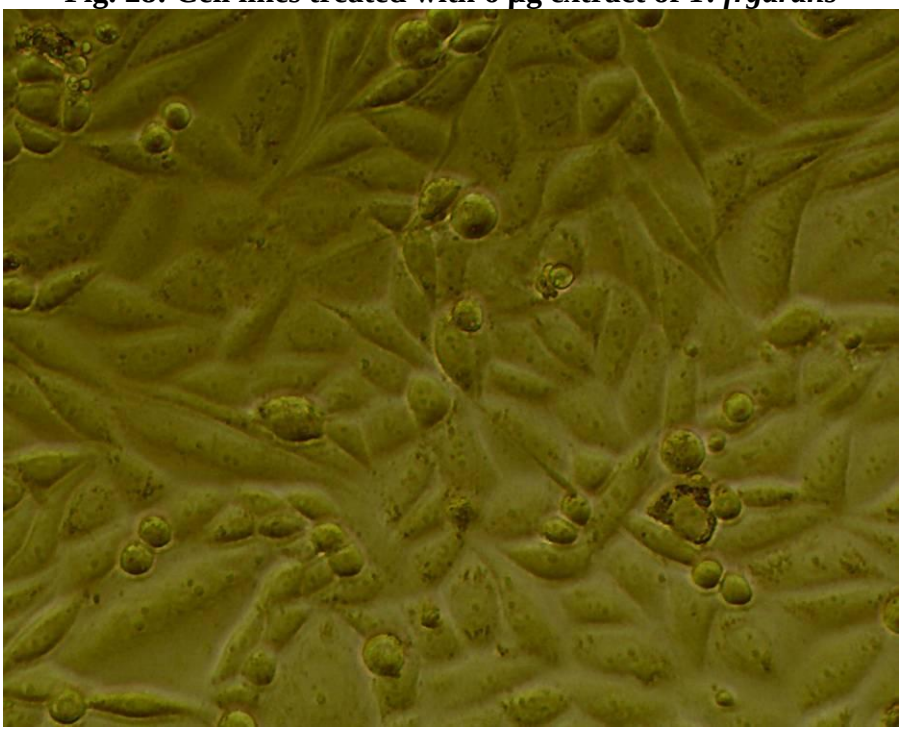

Fig. 29: Cell lines treated with 8 µg extract of *T. frgarans*

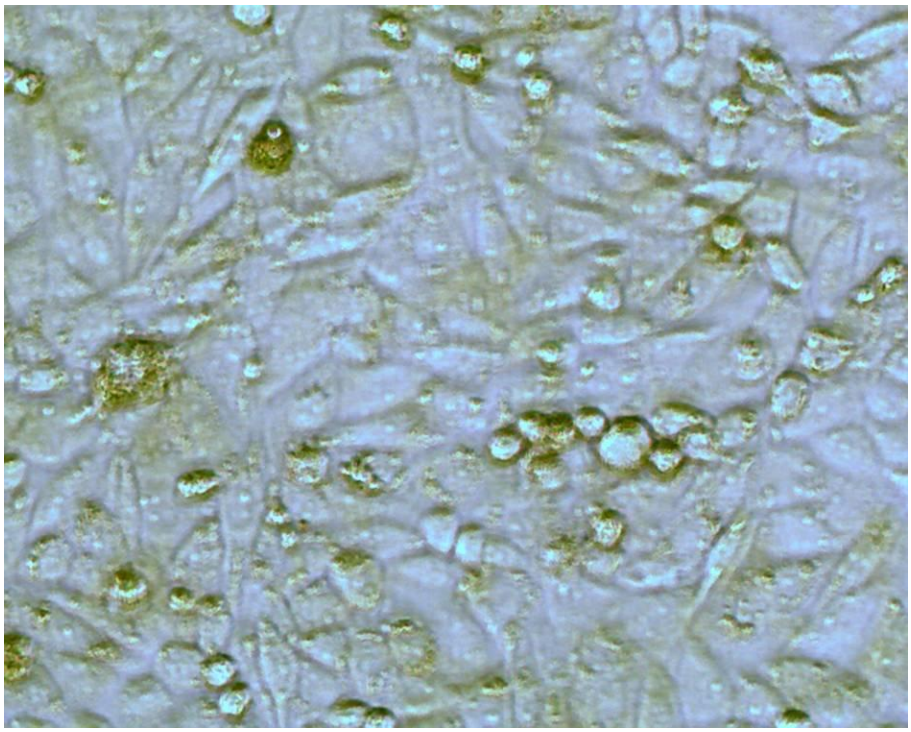

Fig. 30: Cell lines treated with 16 µg extract of *T. frgarans*

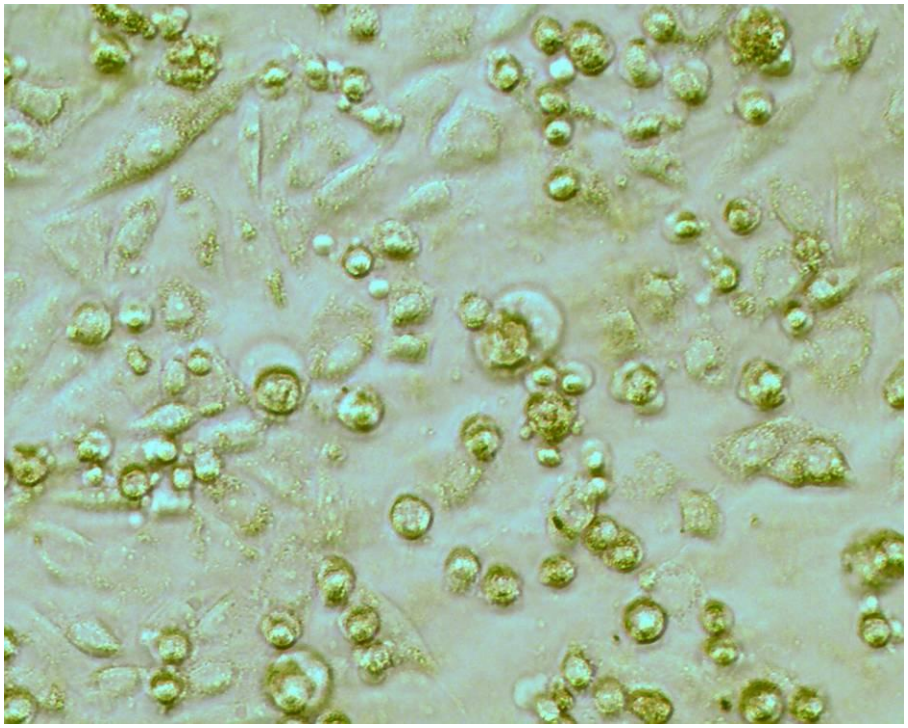

Fig. 31: Cell lines treated with 24µg extract of *T. frgarans*

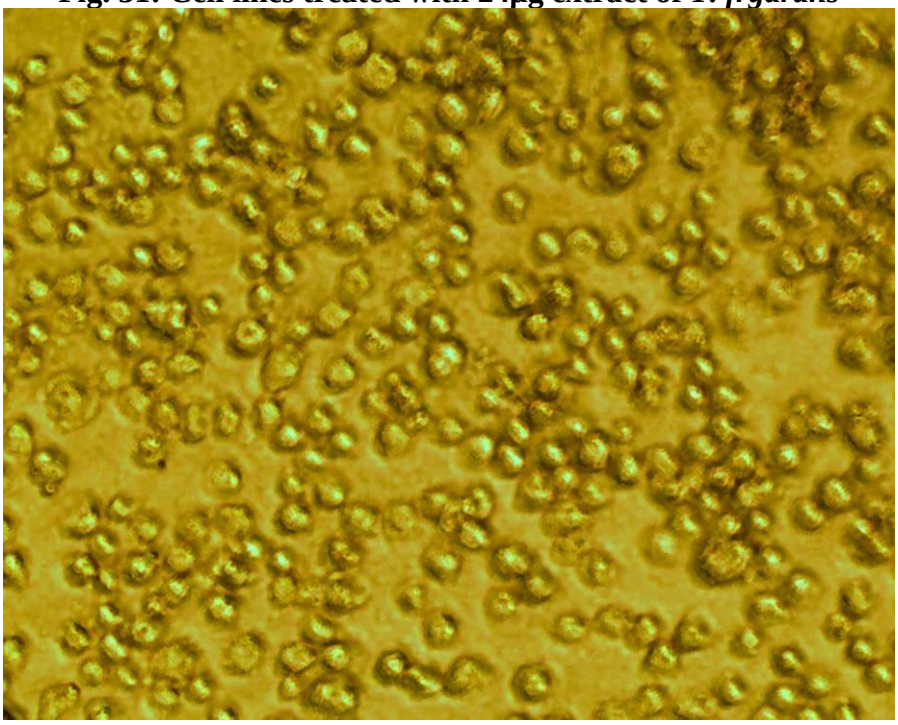

Fig. 32: Cell lines treated with 32µg extract of *T. frgarans*

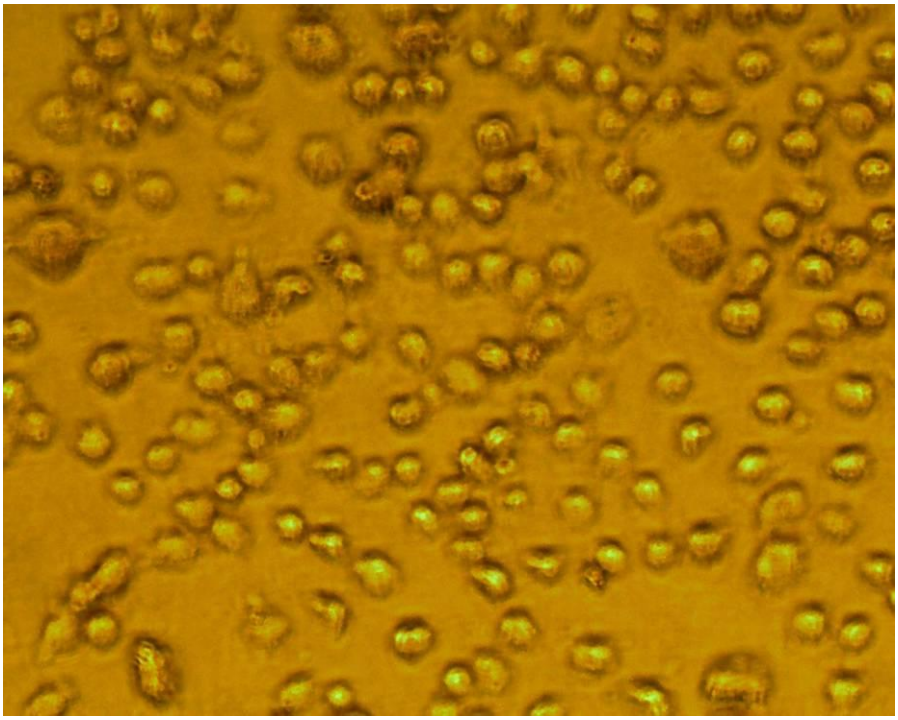

Fig. 33: Cell lines treated with 40µg extract of *T. frgarans*

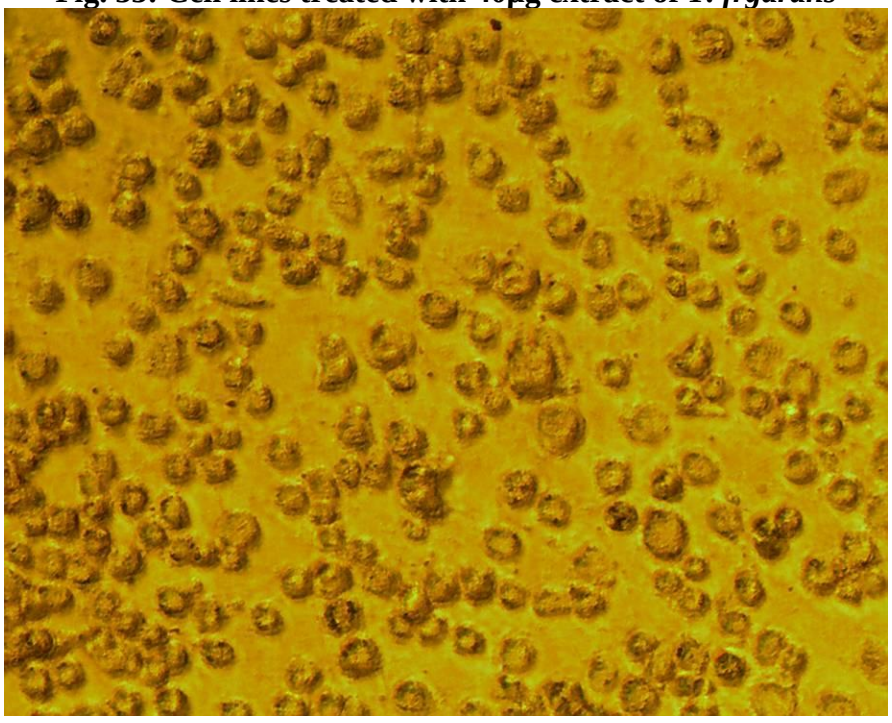

Fig. 35: Antibacterial activity of *T. frgarans* against *Psedumonas aeruginosa*

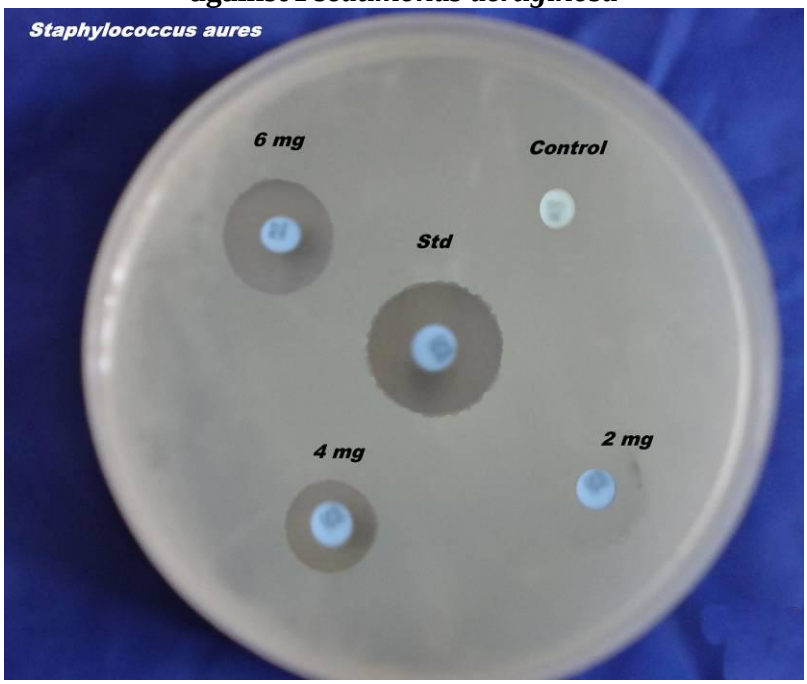

Fig. 36: Antibacterial activity of *T. frgarans* against *Escherichia coli*

Fig. 37: Antibacterial activity of *T. frgarans* against *Klebsiella pneumonia*

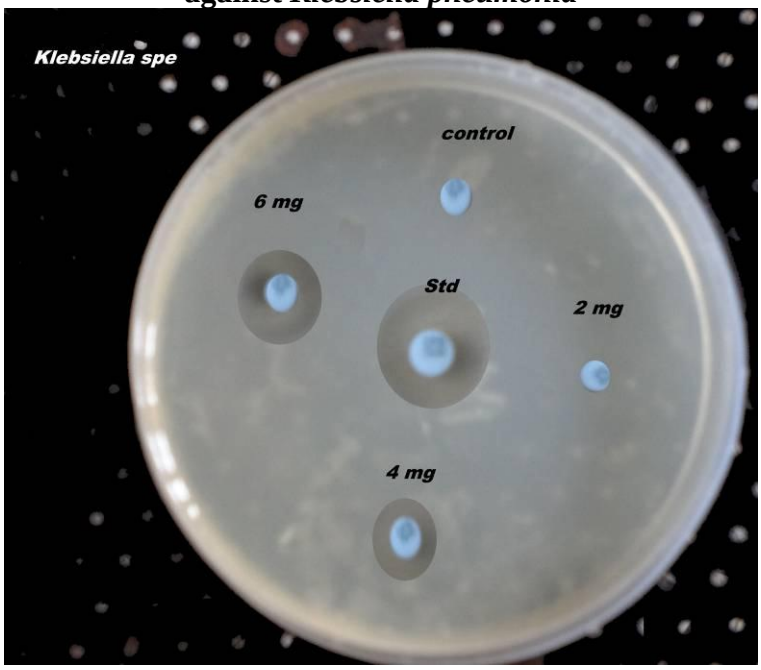

Fig. 38: Antibacterial activity of *T. frgarans* against *Proteus mirbilis*

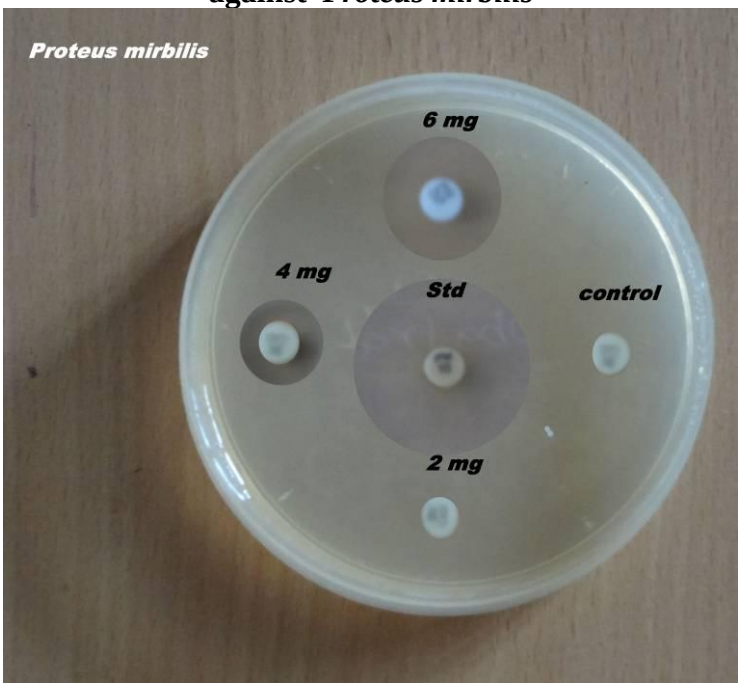

Fig. 39: Antibacterial activity of *T. frgarans* against *Staphylococcus aureus*

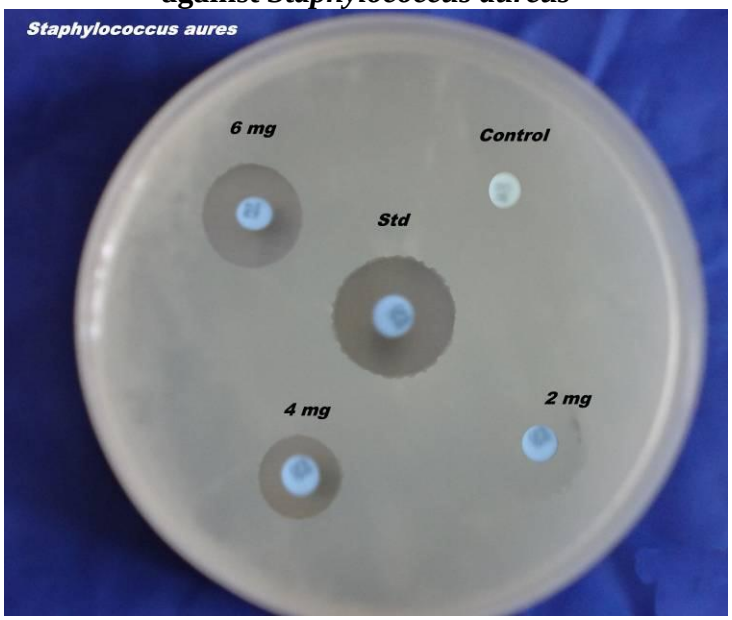

Fig. 40: Antimicrobial activity of *T. frgarans* against *Candida albicans*

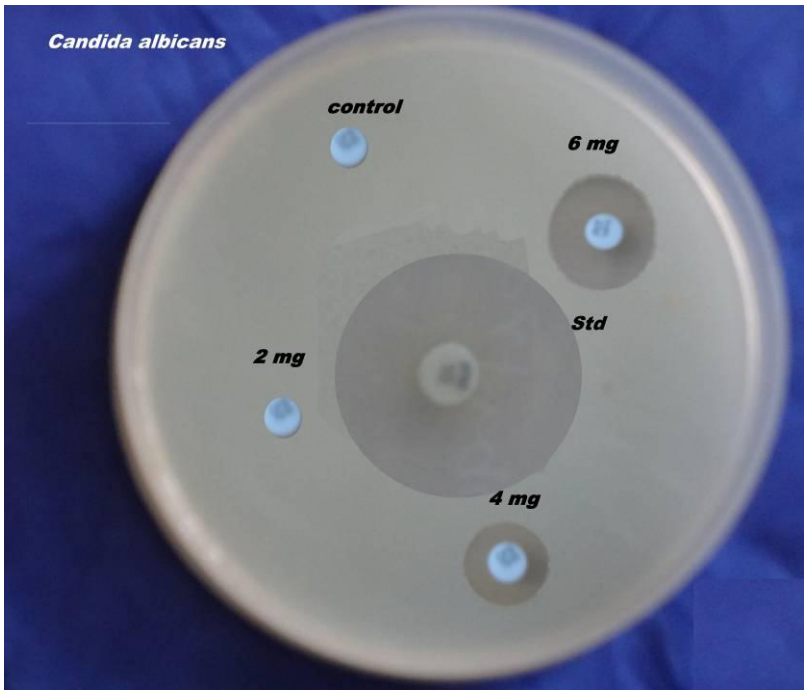

Fig. 1.1: Habitat of *Thunbergia fragrans*

Fig. 1.2: Habitat of *Thunbergia fragrans*

Fig. 1.3*: T. fragrans* flower

Fig. 1.4: *T. fragrans* fruit

Fig 1.5.Seed *T. fragrans*

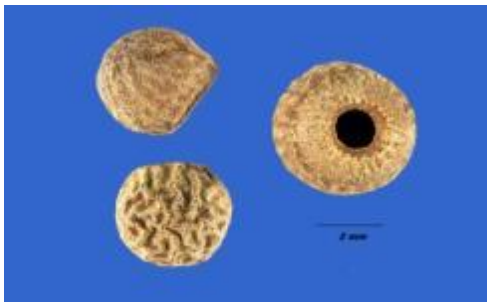

Fig. 1.6: Leaf ventral view

Fig. 1.7 Leaf dorsal view

Fig. 2. 1: T.S of Leaf through midrib

Fig. 2. 2: T.S of Midrib enlarged

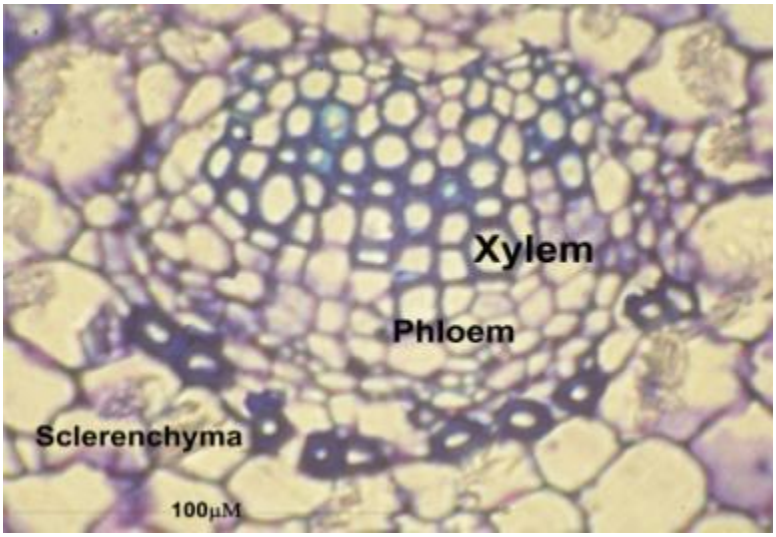

Fig. 3.1: T.S. of lamina

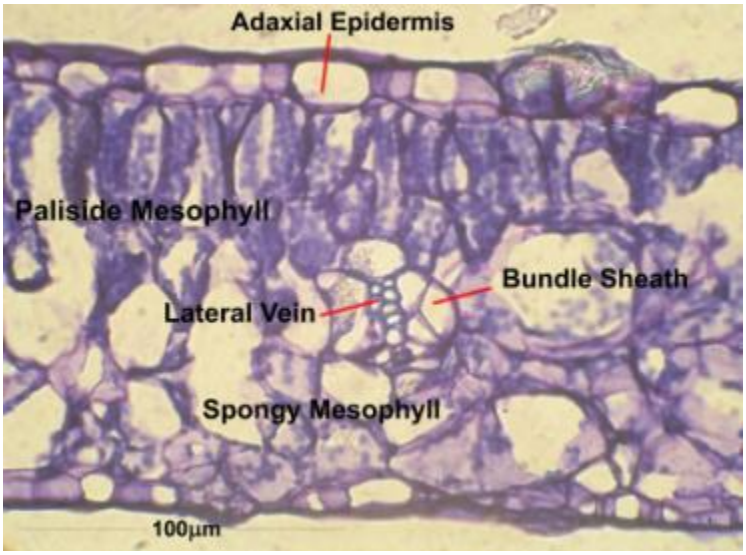

Fig. 3.2: Venation of the lamina

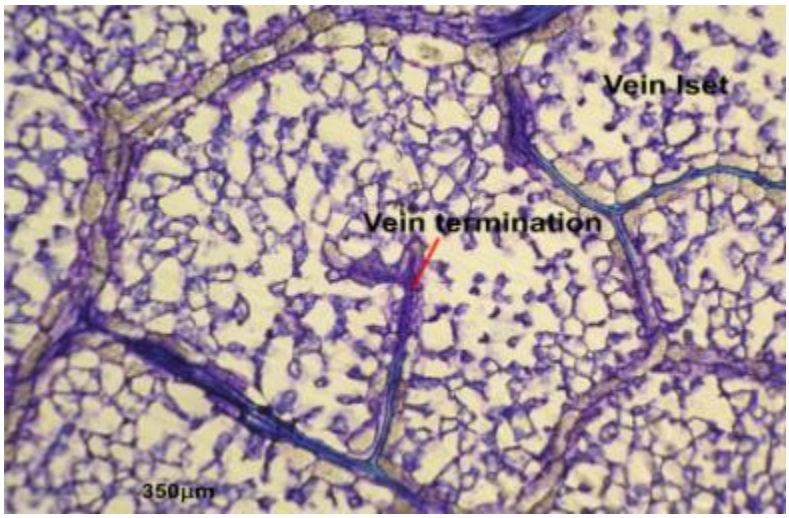

Fig. 4.1: Crystals in Bundle Sheath parenchyma

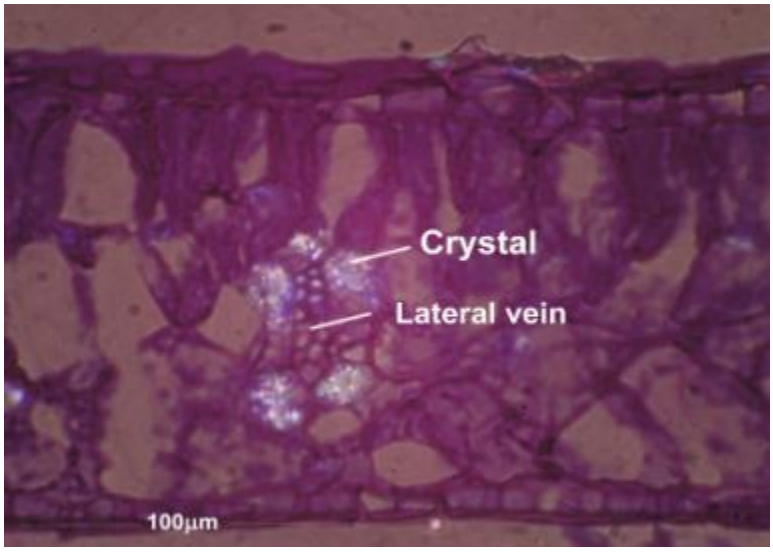

Fig. 4.2: Crystals in the bundle sheath parenchyma

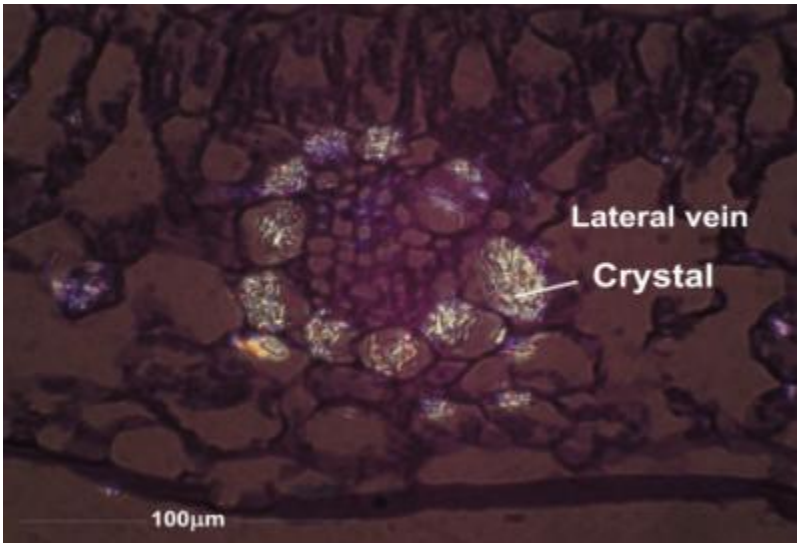

Fig. 4.3: Crystals in the bundle sheath parenchyma

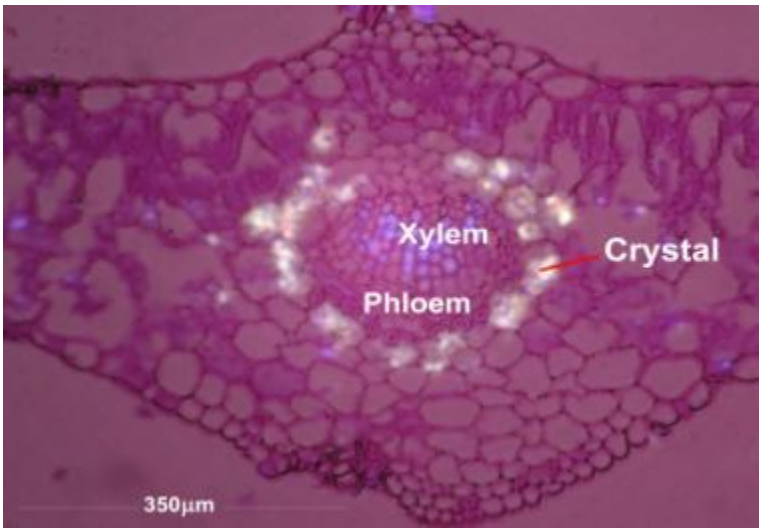

Fig. 5.1: Adaxial Epidermis (Anticlinal walls)

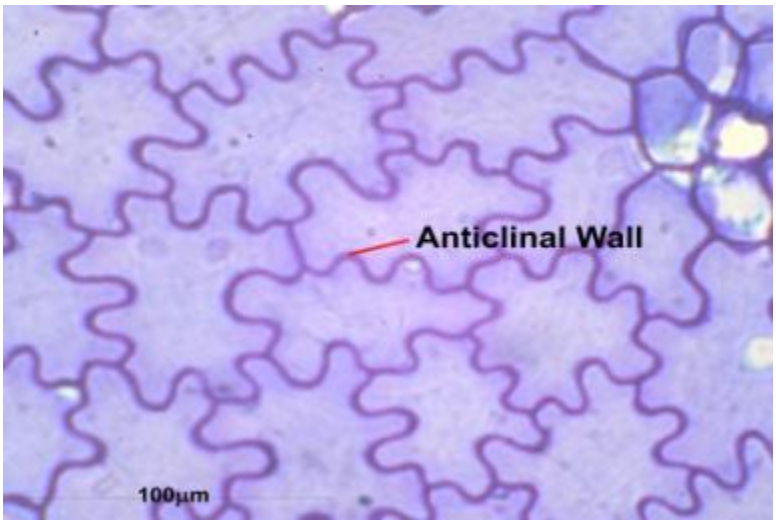

Fig. 5.2: Abaxial Epidermis with stomata

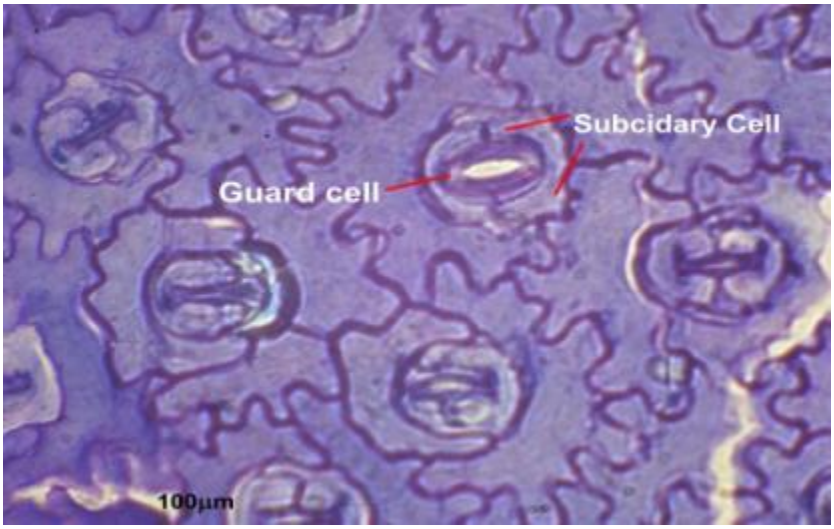

Fig. 5.3: One stomata enlarged

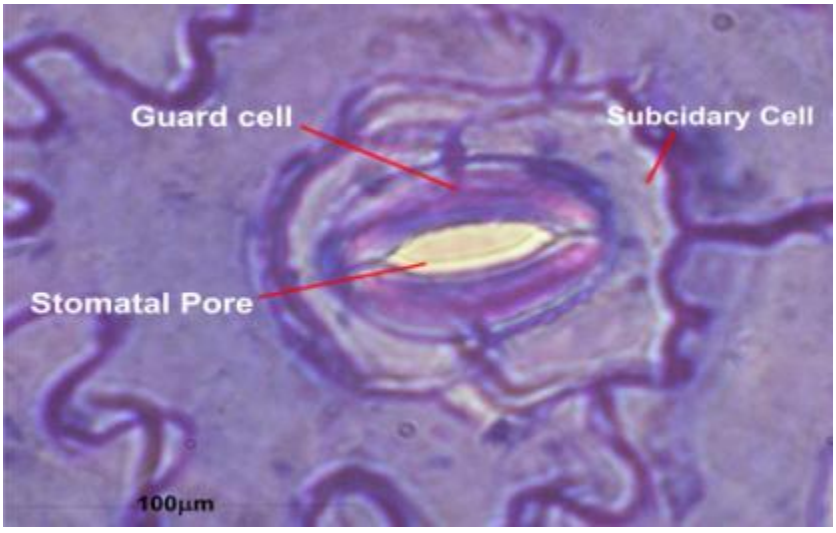

Fig. 6.1: Venation pattern

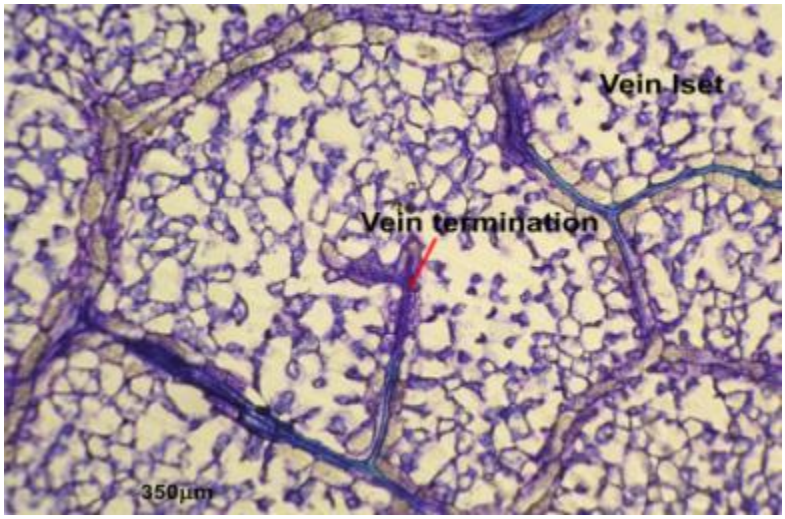

Fig. 6.2: Crystals along the veins

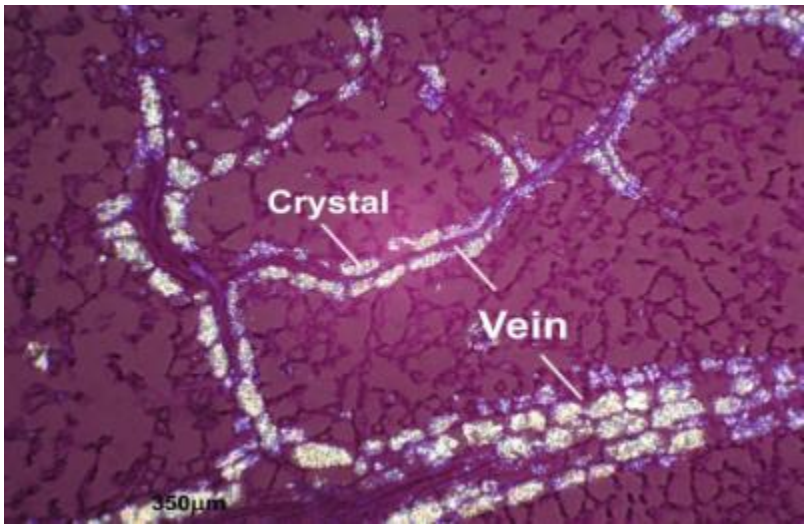

Fig. 6.3: Crystals enlarged

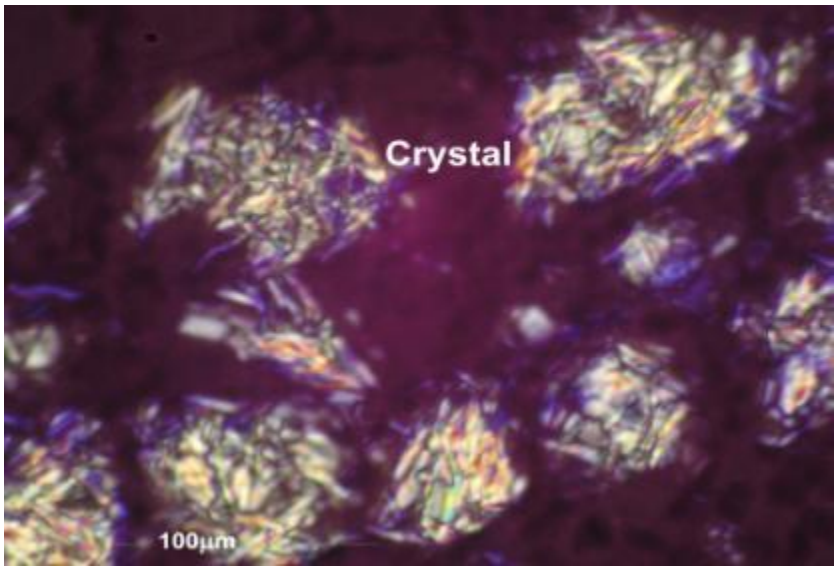

Fig. 7.1: T.S of Petiole

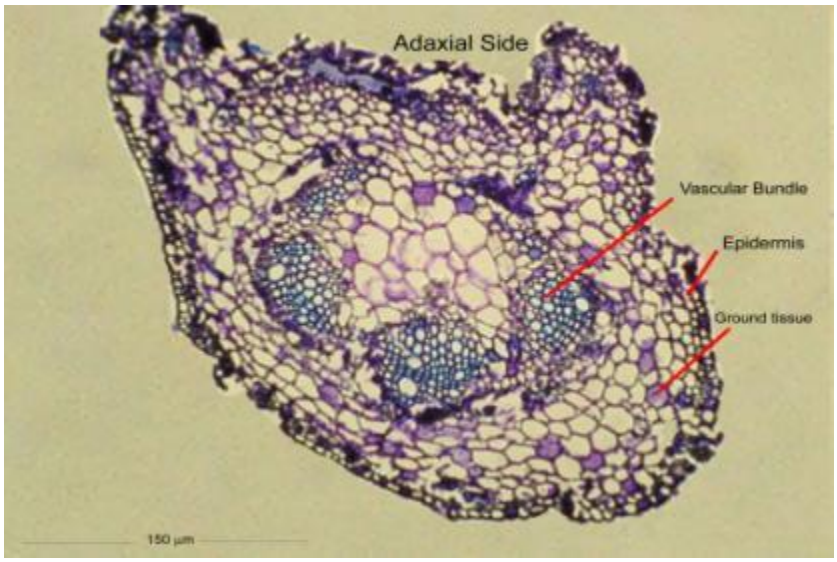

Fig. 7.2: Vasicular Bundles of petiole enlarged

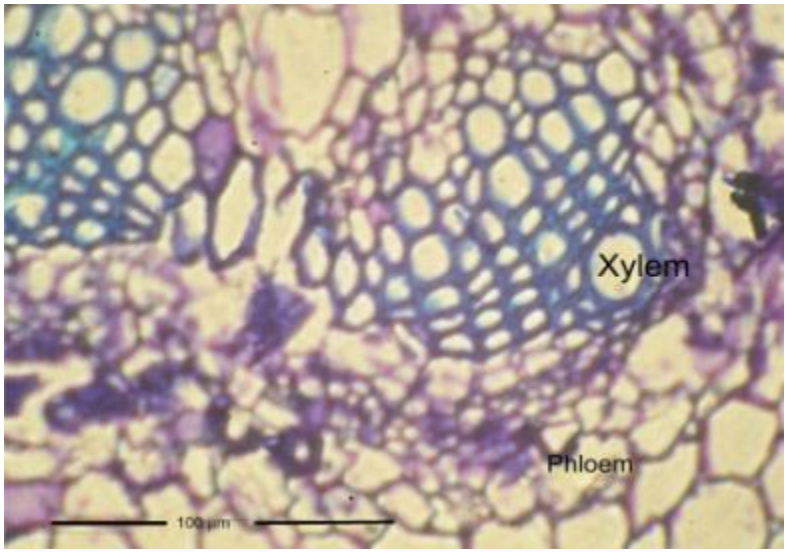

Fig. 8.1: T.S of Stem

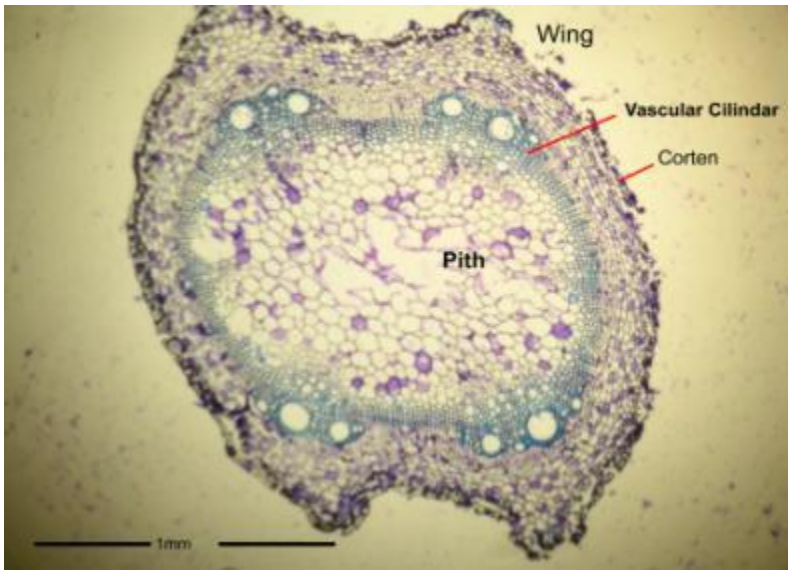

Fig. 8.2: T.S of stem (a sector)

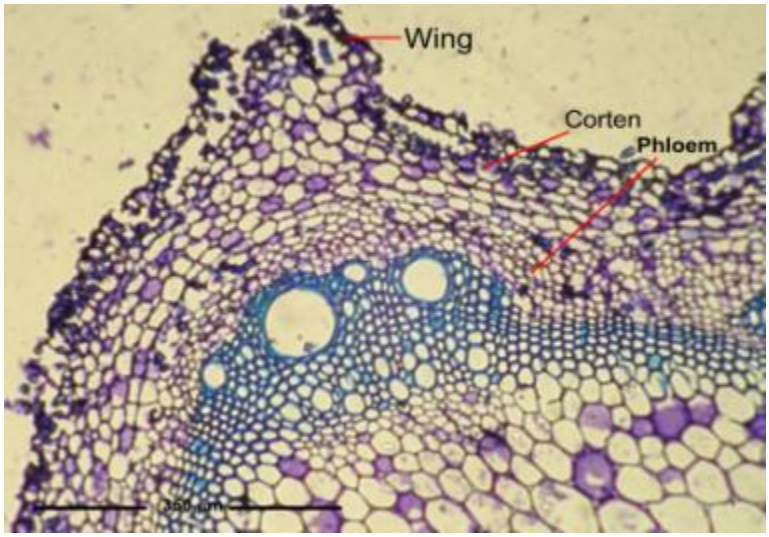

Fig. 8.3: Secondary Xylem

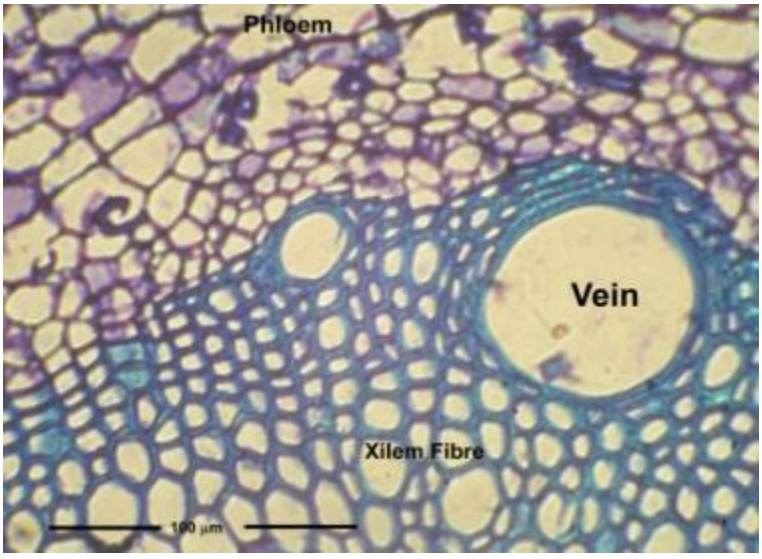

Fig. 9.1: Leaf powder showing adaxial epidermis

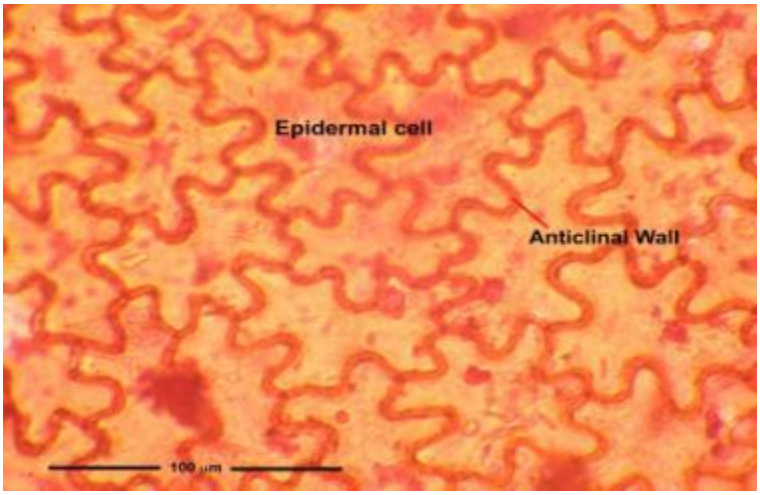

Fig. : 9.2 Leaf powder showing abaxial epidermis

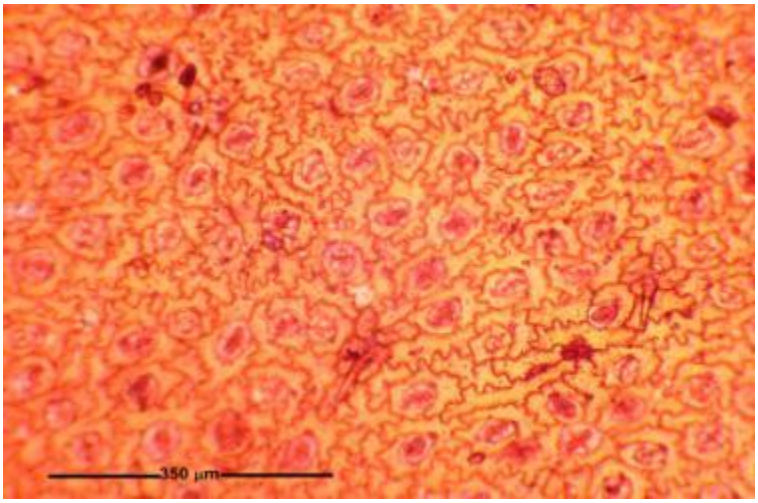

Fig. 9.3: Stomato enlarged

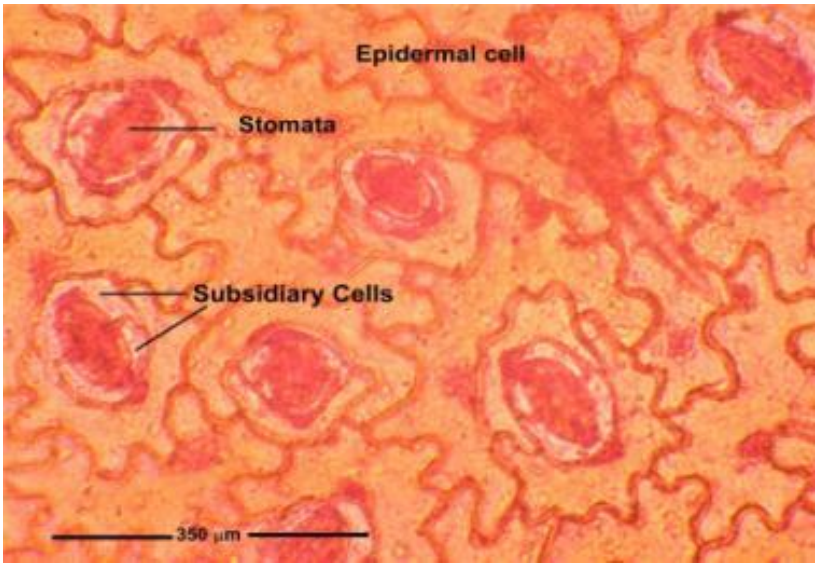

Fig. 10.1: Leaf powder showing epidermal trichomes

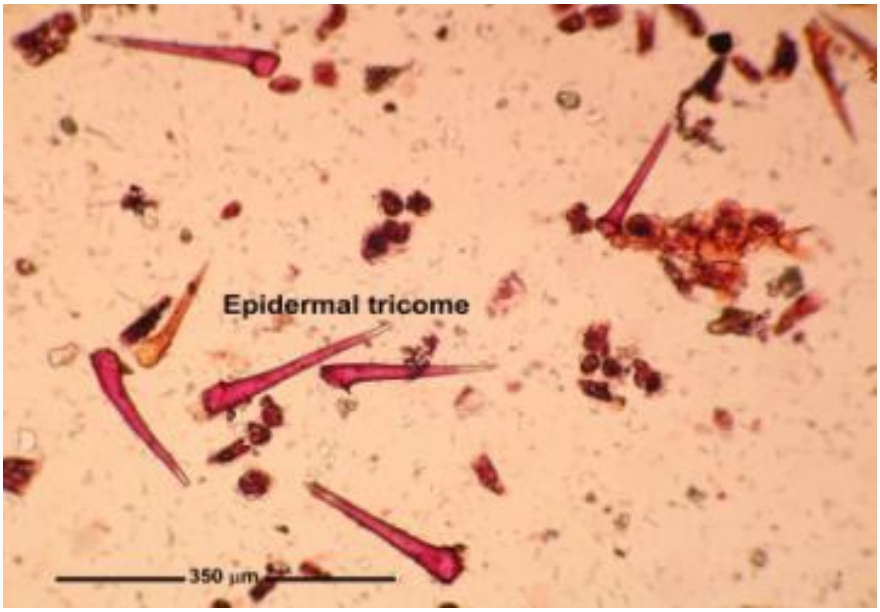

Fig. : 10.2 One trichome enlarged

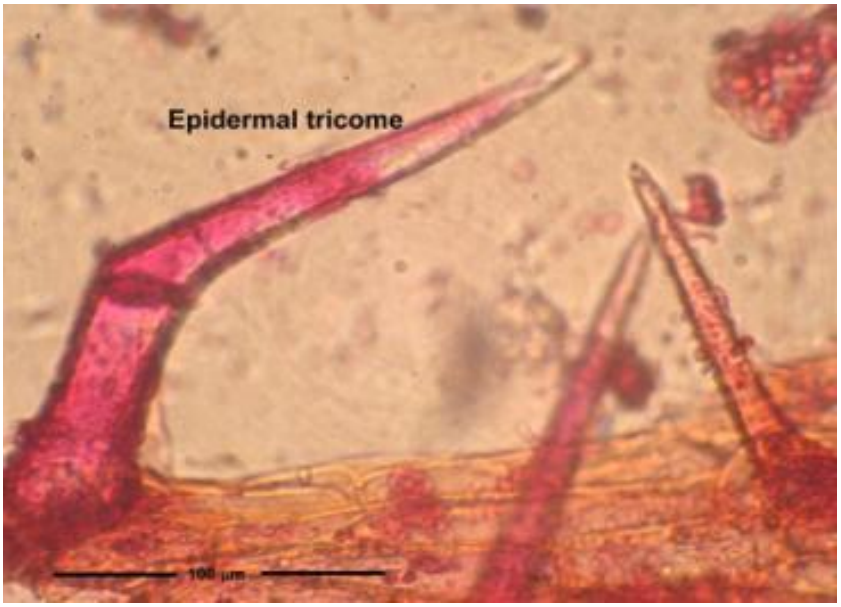

Fig. : 10.3 Crystals in the powder